



CA 02289979 1999-11-12

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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6: <b>A61K 39/00, 39/395, 31/70, C12Q 1/68, C12N 15/55, G01N 33/573</b>	<b>A1</b>	(11) International Publication Number: <b>WO 98/51335</b> (43) International Publication Date: 19 November 1998 (19.11.98)
(21) International Application Number: <b>PCT/CA98/03475</b> (22) International Filing Date: <b>15 May 1998 (15.05.98)</b> (30) Priority Data: 60066,537 15 May 1997 (15.05.97) US 60061,654 11 October 1997 (10.10.97) US (71)(72) Applicant and inventor: <b>LEVY, Gary [CA/CA]; 260 Res- denson Avenue, Thornhill, Ontario L3T 2B2 (CA).</b>	(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GR, GM, GW, HN, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TH, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BA, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, NG, TD, TN).</b>	
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(54) Title: <b>METHODS OF MODULATING IMMUNE COAGULATION</b>		
(57) Abstract <p>Methods for modulating immune coagulation using novel antibodies and compounds are described. A protein Fig2 having direct prothrombinase activity has been identified. Inhibitors of Fig2 are useful in preventing and treating diseases which require a reduction in immune coagulation including bacterial and viral infections, allograft and xenograft rejection, glomerulonephritis, cancer, a number of gastrointestinal diseases and fetal loss.</p>		

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**CLAIM:**

1. A method of preventing graft rejection comprising administering an effective amount of an inhibitor of Fgl2 to an animal in need thereof.
2. A method of preventing or treating fetal loss comprising administering an effective amount of an inhibitor of Fgl2 to an animal in need thereof.
3. A method according to claim 1 or 2 wherein said inhibitor is an antibody that binds to Fgl2.
4. A method according to claim 3 wherein the antibody is a monoclonal antibody that binds to a human Fgl2 having the amino acid sequence as shown in Figure 5.
5. A method according to claim 4 wherein the antibody binds an epitope of human Fgl2 comprising the amino acids at positions 364-378 (DRYP5GNCGLYYSSG) in Figure 5.
6. A method for diagnosing or monitoring graft rejection in an animal comprising detecting a Fgl2 protein or a Fgl2 nucleic acid in a biological sample from the animal.
7. A method for diagnosing or monitoring fetal loss in an animal comprising detecting a Fgl2 protein or a Fgl2 nucleic acid in a biological sample from the animal.
8. A method according to claim 6 or 7 comprising detecting (a) a nucleic acid molecule having a sequence shown in Figure 2 or 3 or SEQ.ID.NO.:1 or 3, or a fragment thereof, or (b) a protein having an amino acid sequence as shown in Figure 5 or 56Q.ID.NO.:2 or 4, or a fragment thereof.
9. A method for detecting a Fgl2 protein according to claim 8 comprising contacting the sample with an antibody that binds to Fgl2 which is capable of being detected after it becomes bound to the Fgl2 in the sample.
10. A method for detecting a nucleic acid molecule encoding Fgl2 according to claim 8 comprising contacting the sample with a nucleotide probe capable of hybridizing with the nucleic acid molecule to form a hybridization product, under conditions which

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permit the formation of the hybridization product, and assaying for the hybridization product

11. A method according to claim 10 further comprising treating the sample with primers which are capable of amplifying the nucleic acid molecule in a polymerase chain reaction to form amplified sequences under conditions which permit the formation of amplified sequences, and assaying for amplified sequences.
12. A method of inducing immune coagulation comprising administering a nucleic acid sequence encoding Fg12 or an Fg12 protein to an animal in need thereof.
13. A method according to claim 12 comprising administering (a) a nucleic acid molecule having a sequence shown in Figure 2 or 3 or SEQ.ID.NO.:1 or 3 or (b) a protein having a sequence shown in Figure 5 or SEQ.ID.NO.:2 or 4.
14. A composition for use in inhibiting graft rejection in an animal comprising (a) an antibody specific for a Fg12 protein or (b) an antisense oligonucleotide to Fg12.
15. A composition for use in inhibiting fetal loss in an animal comprising (a) an antibody specific for a Fg12 protein or (b) an antisense oligonucleotide to Fg12.
16. A method for preventing graft rejection in an animal comprising administering a therapeutically effective amount of a composition as claimed in claim 14.
17. A method for preventing or treating fetal loss in an animal comprising administering a therapeutically effective amount of a composition as claimed in claim 15.
18. A vaccine for preventing graft rejection comprising an effective amount of an Fg12 protein or peptide in admixture with a suitable diluent or carrier.
19. A vaccine for preventing fetal loss comprising an effective amount of an Fg12 protein or peptide in admixture with a suitable diluent or carrier.
20. An isolated nucleic acid molecule comprising (a) the sequence shown in Figure 8, where T can also be U; (b) nucleic acid sequences which have substantial sequence identity with (a); and (c) a fragment of (a) or (b).

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21. An isolated nucleic acid molecule comprising (a) the sequence shown in Figure 4, where T can also be U; (b) nucleic acid sequences which have substantial sequence identity with (a); and (c) a fragment of (a) or (b).

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**Title: Methods of Modulating Immune Coagulation****FIELD OF THE INVENTION**

The present invention relates to methods for modulating immune coagulation using novel antibodies and compounds that modulate immune coagulation.

5 **BACKGROUND OF THE INVENTION**

Activation of the coagulation pathways is an important part of immune and inflammatory reactions and is associated with bacterial and viral infections (e.g. endotoxin shock, viral hepatitis), glomerulonephritis (GN), cancer, a number of gastrointestinal diseases, allograft and xeno graft rejection and spontaneous or stress-triggered fetal loss.

- 10 Immune coagulation is mediated by a number of coagulants that, when triggered, activate specific ligands resulting in cleavage and activation of coagulation pathways that lead to fibrin deposition. The molecular events leading to expression of immune coagulants involve natural antibodies binding both to antigens on endothelial cells and Fc receptors on macrophages and endothelial cells. An additional mechanism is immune
- 15 complex-mediated induction of macrophage procoagulants. These events lead to thrombin production which initiates platelet activation and ultimately fibrin deposition.

- In 50% of hepatitis patients moderate to severe consumptive coagulopathy, or disseminated intravascular coagulopathy is found associated with fulminant hepatitis. Thrombi formation is observed around necrotic areas (Sinclair et al., 1990 and Lee, W. M.,
- 20 1993). As a consequence of hepatitis, levels of factors II, V, VII, and X are decreased in the liver, reflecting both consumptive coagulopathy and a decrease in hepatic synthetic function. Also, the levels of thrombin-antithrombin complexes are high and platelet counts are low (Lee, W. M., 1993). These results indicate that the host immune system, including the coagulation pathway, is disrupted as a result of HBV infection. The limited host range
- 25 of HBV and the difficulty to propagate the virus in tissue culture have hampered the understanding of HBV and hepatitis B.

- Mononuclear phagocytes and macrophages are implicated in the pathogenesis of hepatitis specific induction of procoagulant activity because of their role in coagulation; they synthesize some of the essential coagulation factors such as tissue factor
- 30 and their surfaces serve as sites of fibrin deposition. Factors participating in the coagulation cascade are released as inactive zymogens and upon activation, by preceding activated factors, they are converted to their active form. The factors are predominantly serine proteases (Davie et al., 1991). Factors VIIa, XIIa, XIa, Xa, IXa, thrombin, kallikrein, and plasminogen are categorized under family 1 serine proteases (Davie et al.,
- 35 1991; Barrett and Rawlings, 1995; Rawlings and Barrett, 1994; Nduwimana et al., 1995). In order to initiate the coagulation cascade the procoagulants need to be expressed. Ruegg and Pytela, 1995 isolated a cDNA encoding a protein that is homologous to a murine

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fibrinogen-like protein (Koyama et al. 1987). However, they did not determine the function of the protein or realize its use in modulating immune coagulation.

In view of the many diseases associated with the activation of the coagulation pathways, there is a need to identify and characterize procoagulants and to develop methods for modulating immune coagulation that are useful in the prevention, treatment and diagnosis of diseases associated therewith including bacterial and viral infections, glomerulonephritis (GN), cancer, a number of gastrointestinal diseases, allograft and xenograft rejection and spontaneous or stress-triggered fetal loss.

#### SUMMARY OF THE INVENTION

The present inventor has identified and characterized an immune procoagulant, and the molecular and cellular events leading to its production. Specifically, the mouse and human direct prothrombinase genes (referred to herein as "mFg12" and "hFg12" respectively) have been cloned and sequenced. The nucleic acid sequence of the human and mouse Fg12 is shown in SEQ.ID.NO.1 and 3, respectively. The genes encode a transmembrane serine protease which has functional prothrombinase activity. The proteins encoded by the genes have been sequenced in both humans and mice. The protein has a molecular weight of approximately 70kd. The hFg12 gene has been mapped to chromosome 7 and the mFg12 gene to chromosome 5. The inventor has cloned and sequenced the genomic DNA encoding the human prothrombinase. The organization of the genomic DNA encoding hFg12 is shown schematically in Figure 1. The nucleic acid sequence of the promoter region, exon 1, exon 2 and the 3' UTR are shown in Figures 8, 2, 3 and 4, respectively. The amino acid sequence of the human and mouse Fg12 protein is shown in Figure 5 and in SEQ.ID.NO.2 and 4, respectively.

The determination by the inventor that Fg12 is a direct prothrombinase allows the development of diagnostic methods and therapies for conditions involving immune coagulation.

Accordingly, the present invention provides a method of inhibiting immune coagulation comprising inhibiting the activity or expression of Fg12. The method can be used *in vivo* to treat a condition which requires a reduction in immune coagulation such as bacterial and viral infections, glomerulonephritis (GN), cancer, a number of gastrointestinal diseases, allograft and xenograft rejection and fetal loss.

In one aspect, the activity of Fg12 may be inhibited using an antibody that binds to Fg12. The present inventor has developed a panel of monoclonal and polyclonal antibodies which neutralize Fg12 and prevent the fibrin deposition associated with endotoxin shock, viral hepatitis, allograft and xenograft rejection. The antibodies were shown to prevent cellular infiltration, fibrin deposition and tissue damage, and lead to enhanced survival. In particular, antibodies against the direct prothrombinase (Fg12) were found to be extremely useful in preventing diseases known to have associated massive fibrin

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deposition and coagulative necrosis, including allograft and xenograft rejection as well as fetal loss induced by stress or cytokines.

In one embodiment, the present invention provides a method of preventing or reducing graft rejection comprising administering an effective amount of an antibody to  
5 Fgl2 to an animal in need thereof.

In another embodiment, the present invention provides a method of preventing or reducing fetal loss comprising administering an effective amount of antibody to Fgl2 to an animal in need thereof.

Antibodies can be prepared using entire Fgl2 proteins or immunogenic  
10 portions thereof. Preferably, such portions bind with an affinity of at least about  $10^6$  L./mole to an antibody raised against Fgl2. In particular, the present inventor has shown that a peptide comprising amino acid residues 300 to 400 is useful in raising antibodies. Accordingly, the present invention contemplates antibodies which (a) immunoreact with  
15 peptides comprising the amino acids at approximately positions 300 to 400 in Figure 5; and (b) neutralize the prothrombinase activity of hFgl2. The invention also relates to hybridoma cell lines that produce the monoclonal antibodies, and inhibitors and activators thereof.

In another aspect, the expression of Fgl2 may be inhibited using antisense molecules that are complementary to a nucleic acid sequence from the Fgl2 gene. In  
20 particular, the nucleic acid sequences for Fgl2 as shown in Figures 2 or 3 may be inverted relative to their normal presentation for transcription to produce antisense nucleic acid molecules.

Additional inhibitors of Fgl2 may be identified by testing substances that inhibit the prothrombinase activity of Fgl2. In particular, the invention contemplates a  
25 method for assaying for a substance that affects the prothrombinase activity of Fgl2 comprising (a) reacting Fgl2, a substrate which is capable of being cleaved by Fgl2 to produce a product, and a test substance, under conditions which permit cleavage of the substrate to produce the product; (b) assaying for product; and (c) comparing to the product  
30 obtained in the absence of the substance to determine the effect of the substance on the prothrombinase activity of the Fgl2 protein.

The nucleic acid molecules encoding Fgl2, Fgl2 proteins, and monoclonal antibodies of the present invention have diagnostic and monitoring applications. In particular they may be used in conventional assays to monitor or diagnose conditions such as bacterial and viral infections (e.g. endotoxin shock, viral hepatitis), allograft rejection,  
35 glomerulonephritis, cancer, a number of gastrointestinal diseases and fetal loss.

In one embodiment, the present invention provides a method for diagnosing or monitoring a condition involving increased immune coagulation in an animal comprising detecting a Fgl2 protein or a Fgl2 nucleic acid in a biological sample from the animal.

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The invention also contemplates compositions comprising, and methods of using (a) the monoclonal antibodies produced by the hybridoma cell lines of the invention; (b) inhibitors and activators of the monoclonal antibodies; (c) antibodies to a Fgl2; (d) antisense nucleic acid molecules to *fgl2*; and (e) substances identified using the methods of the invention (e.g. inhibitors and activators of the expression of a nucleic acid molecule of the invention; and, inhibitors and activators of the activity of a Fgl2 protein of the invention).

The compositions of the invention may be used in the prevention or treatment of conditions requiring a reduction in procoagulant activity. Therefore, the invention contemplates a composition for treating a condition requiring a reduction in procoagulant activity comprising administering a therapeutically effective amount of one or more inhibitors of Fgl2. The inhibitor may be an antibody specific for a Fgl2; an antisense nucleic acid molecule of the invention; substances identified in accordance with the methods of the invention or a monoclonal antibody produced by a hybridoma cell line of the present invention. Conditions which require reduction in procoagulant activity include bacterial and viral infections (e.g. endotoxin shock, viral hepatitis), allograft and xenograft rejection, glomerulonephritis, cancer, a number of gastrointestinal diseases and fetal loss.

The present invention also contemplates a vaccine for preventing graft rejection comprising an amount of a Fgl2 protein which is effective to provide protection against graft rejection.

The present invention also contemplates a vaccine for preventing fetal loss comprising an amount of a Fgl2 protein which is effective to provide protection against fetal loss.

Other objects, features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

#### DESCRIPTION OF THE DRAWINGS

The invention will be better understood with reference to the drawings in which:

Figure 1 is a schematic representation of the *hfgl2* gene;

Figure 2 shows the nucleotide sequences of exon 1 of the mouse and human *Fgl3* genes;

Figure 3 shows the nucleotide sequences of exon 2 of the mouse and human

*Fgl2* genes;

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Figure 4 shows the nucleotide sequence of the 3' UTR of hFg12;

Figure 5 shows the amino acid sequences of the mouse and human Fg12 proteins with the serine protease sites boxed;

Figure 6 is the amino acid sequence of the mouse and human Fg12 proteins, with the 5 glycosylation sites underlined;

Figure 7 shows the predicted secondary structure of the hFg12 protein;

Figure 8 shows the nucleotide sequence of the mouse and human Fg12 gene promoter regions;

Figure 9 shows the nucleic acid sequence of the transcription binding sites in the putative promoter region of hfg12;

Figure 10A is a sample of electrophoresis of PAC clones on a CHR gel;

Figure 10B is a sample of electrophoresis of PAC clones on regular gel;

Figure 11 is a restriction map of three PAC clones;

Figure 12 is a graph showing the prevention of C5A graft rejection by C5A alone or in combination with antibodies to immune coagulants.

Figure 13 is a map of the pGL2-Basic -fgf-2 Promoter Region Constructs.

Figure 14 shows Fgf-2 induction in xenoserum versus autologous serum.

Figure 15 shows dose response curves for fgf-2 induction in xenoserum versus autologous serum.

Figure 16 shows FBS induction of luciferase activity for the 5' deletion series and pL3'274.

Figure 17 shows the Fgf-2 promoter DNA sequence.

Figure 18 is a graph showing the prevention of fetal loss by monoclonal antibody 3D4.3.

Figure 19 is a gel showing the time course of expression of murine fg12.

Figure 20 is a Western blot showing expressed fg12.

Figure 21 is a Coomassie blue stained gel showing expressed fg12.

Figure 22 is an autoradiograph showing 125I labelled fg12.

#### **DETAILED DESCRIPTION OF THE INVENTION**

The following standard abbreviations for the amino acid residues are used throughout the specification: A, Ala - alanine; C, Cys - cysteine; D, Asp - aspartic acid; E, Glu - glutamic acid; F, Phe - phenylalanine; G, Gly - glycine; H, His - histidine; I, Ile - isoleucine; K, Lys - lysine; L, Leu - leucine; M, Met - methionine; N, Asn - asparagine; P, Pro - proline; Q, Gln - glutamine; R, Arg - arginine; S, Ser - serine; T, Thr - threonine; V, Val - valine; W, Trp - tryptophan; Y, Tyr - tyrosine; and p.Y., P.Tyr - phosphotyrosine.

As hereinbefore mentioned, the inventor has cloned and sequenced the human and mouse genes encoding the protein Fg12. The inventor has characterised the Fg12 proteins and has shown that it is a direct prothrombinase. The determination by the is a

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direct prothrombinase allows the development of therapeutic and diagnostic methods and compositions for conditions involving immune coagulation.

## 1. THERAPEUTIC APPLICATIONS

#### (A) Methods of Inhibiting Immune Congulation

5 In one aspect, the present invention includes methods of inhibiting immune coagulation by inhibiting the activity or expression of Fgl2. Methods that inhibit immune coagulation may be useful in treating conditions which require reduction in procoagulant activity including bacterial and viral infections (e.g. endotoxin shock, viral hepatitis), allograft and xenograft rejection, glomerulonephritis, cancer, a number of gastrointestinal  
10 diseases and fetal loss.

Accordingly, the present invention provides a method of preventing or treating a condition requiring a reduction in immune coagulation comprising administering an effective amount of an inhibitor of Fg12 to an animal in need thereof.

Administration of an "effective amount" of the compounds of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. The effective amount of a compound of the invention (such as an inhibitor of Fgl2) may vary according to factors such as the disease state, age, sex, and weight of the animal. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. The term "animal" as used herein includes all members of the animal kingdom, including humans. Preferably, the animal to be treated is a human.

Inhibitors of Fgl2 include substances that inhibit the transcription and translation of the *Fgl2* gene as well as substances that inhibit the prothrombinase activity of the Fgl2 protein.

### (i) Antibodies

Examples of substances that can inhibit the prothrombinase activity of the FgI2 protein are polyclonal and monoclonal antibodies that bind and neutralize FgI2.

Accordingly, the present invention provides a method of preventing or  
33 treating a condition requiring a reduction in immune coagulation comprising administering  
an effective amount of an antibody to Fgl2 to an animal in need thereof. An effective  
amount of an antibody means an amount of the antibody that is effective to neutralize or  
inhibit the prothrombinase activity of the Fgl2 protein.

The inventor has prepared monoclonal antibodies that neutralize the  
35 activity of Fgl2. In particular, the inventor has shown that antibodies to Fgl2 can inhibit  
graft rejection in both allograft and xenograft models. Therefore, the present invention  
provides a method of preventing or reducing graft rejection comprising administering an

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effective amount of an antibody to Fgf2 to an animal in need thereof. In one embodiment, the animal is a human and the antibody binds human Fgf2.

The inventor has also shown that antibodies to Fg12 can prevent or reduce fetal loss resulting from stress or cytokines. Therefore, the present invention also provides a method of preventing or reducing fetal loss comprising administering an effective amount of an antibody to Fg12 to an animal in need thereof.

The present invention also provides an antibody that binds an epitope of hFg12 comprising the amino acids at positions 300 to 400 in Figure 5. In a preferred embodiment, the present invention provides an antibody that binds an epitope of hFg12 comprising the amino acids at positions 364-378 (DRYPSNCGLYSSG) in Figure 5.

Antibodies that bind Fg12 can be prepared using techniques known in the art such as those described by Kohler and Milstein, *Nature* 256, 495 (1975) and in U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,611,993 which are incorporated herein by reference. (See also *Monoclonal Antibodies, Hybridomas: A New Dimension in Biological* 15 *Analyses*, Plenum Press, Kennell, McKeown, and Bechtol (eds.), 1980, and *Antibodies: A Laboratory Manual*, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and  $F(ab')_2$ ) and recombinantly produced binding partners. Antibodies are understood to be reactive against the protein encoded by the nucleic acid molecule of the invention if they bind to Fg2 with an affinity of greater than or equal to  $10^{-6}$  M. As will be appreciated by one of ordinary skill in the art, antibodies may be developed which not only bind to the protein, but which bind to a regulator of the protein, and which also block the biological activity of the protein.

Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, a Fgl2 protein of the invention or portions thereof, may be used to immunize an animal. A preferred portion of the protein includes amino acid residues 300 to 400, more preferably 364-378, shown in Figure 5. An animal may be immunized through intraperitoneal, intramuscular, intrasocular, or subcutaneous injections, in conjunction with an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to the protein. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to the protein, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

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Monoclonal antibodies may also be readily generated using conventional techniques as described herein. Generally, hybridoma cell lines are prepared by a process involving the fusion under appropriate conditions of an immortalizing cell line and spleen cells from an animal appropriately immunized to produce the desired antibody.

- 5   Immortalizing cell lines may be murine in origin however, cell lines of other mammalian species may be employed including those of rat, bovine, canine, human origin, and the like. The immortalizing cell lines are most often of tumor origin, particularly myeloma cells but may also include normal cells transformed with, for example, Epstein Barr Virus. Any immortalizing cell may be used to prepare the hybridomas of the present invention.

- 10   Antibody producing cells may be employed as fusion partners such as spleen cells or peripheral blood lymphocytes. The animal from which the cells are to be derived may be immunized at intervals with peptides derived from Fg12. By way of example, animals may be immunized with peptides comprising the amino acids at approximately position 300 to 400 preferably positions 364 to 378 in Figure 5.

- 15   The immortalizing cells and lymphoid cells may be fused to form hybridomas according to standard and well-known techniques employing polyethylene glycol as a fusing agent. Alternatively, fusion may be accomplished by electrofusion.

- Hybridomas are screened for appropriate monoclonal antibody secretion by assaying the supernatant or protein purified from the ascites for reactivity using the method described herein. The hybridomas are screened for antibodies which have the desired properties e.g. neutralize the prothrombinase activity of Fg12.

- 20   The monoclonal antibodies produced by the hybridoma cell lines of the invention are also part of the present invention. In accordance with an embodiment of the invention, the monoclonal antibodies immunoreact with peptides comprising the amino acids at positions 300 to 400 preferably 364 to 378 in Figure 5.

- Monoclonal antibodies which immunoreact with peptides comprising the amino acids at positions 300 to 400 in Figure 5, include homogeneous populations of immunoglobulins. It is understood that immunoglobulins may exist in acidic, basic, or neutral form depending on their amino acid composition and environment, and they may be found in association with other molecules such as saccharides or lipids. The monoclonal antibodies produced by hybridoma cell lines of the invention may be directed against one or more of epitopes of Fg12. Any characteristic epitope associated with Fg12 may provide the requisite antigenic determinant. It is contemplated that monoclonal antibodies produced by the hybridoma cell lines fall within the scope of the present invention so long as they remain capable of selectively reacting with peptides from Fg12 preferably the peptides comprising the amino acids at approximately positions 300-400, most preferably 364 to 378 in Figure 5.

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The antigens recognized by the monoclonal antibodies described herein are also a part of the present invention. An antigen recognized by a monoclonal antibody produced by a hybridoma cell line of the invention, may be localized to specific cells and tissues using conventional immunocytochemistry methods. Cryostat sections may be incubated with a monoclonal antibody of the invention and processed by the avidin-biotin-peroxidase technique (ABC Vectastain). This will determine which class of cells express an antigen of Fg12.

The invention also provides a method for assaying for the presence of an activator or inhibitor of a monoclonal antibody to Fg12 produced by hybridoma cell lines of the invention comprising mixing macrophages, a known concentration of the monoclonal antibody, and a suspected activator or inhibitor of the monoclonal antibody, and assaying for procoagulant activity. The methods of the invention permit the identification of potential stimulators or inhibitors of procoagulant activity.

The present invention includes recombinant or chimeric antibody molecules. Such antibodies or binding partners may be constructed utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or may be purchased from commercially available sources. Primers for mouse and human variable regions including, among others, primers for V<sub>H1a</sub>, V<sub>H1b</sub>, V<sub>H1c</sub>, V<sub>H1d</sub>, C<sub>H1</sub>, V<sub>L</sub>, and C<sub>L</sub> regions are available from Stratagene (La Jolla, Calif). These primers may be utilized to amplify heavy or light chain variable regions, which may then be inserted into vectors such as ImmunoZAP<sup>®</sup> H or ImmunoZAP<sup>®</sup> L (Stratagene), respectively. These vectors may then be introduced into *E. coli* for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the VH and VL domains may be produced (See Bird et al., Science 242:423-426, 1988). In addition, such techniques may be utilized to produce a "human" antibody, without altering the binding specificity of the antibody.

#### (ii) Antisense molecules

Antisense oligonucleotides that are complementary to a nucleic acid sequence from a Fg12 gene can also be used in the methods of the present invention to inhibit Fg12 activity.

Accordingly, the present invention provides a method of preventing or treating a condition requiring a reduction in immune coagulation comprising administering an effective amount of an antisense oligonucleotide that is complementary to a nucleic acid sequence from an Fg12 gene to an animal in need thereof.

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The term "antisense oligonucleotide" as used herein means a nucleotide sequence that is complimentary to its target.

In one embodiment of the invention, the present invention provides an antisense oligonucleotide that is complimentary to a nucleic acid molecule having a sequence as shown in Figure 2 and Figure 3, wherein T can also be U, or a fragment thereof.

The term "oligonucleotide" refers to an oligomer or polymer of nucleotide or nucleoside monomers consisting of naturally occurring bases, sugars, and intersugar (backbone) linkages. The term also includes modified or substituted oligomers comprising non-naturally occurring monomers or portions thereof, which function similarly. Such modified or substituted oligonucleotides may be preferred over naturally occurring forms because of properties such as enhanced cellular uptake, or increased stability in the presence of nucleases. The term also includes chimeric oligonucleotides which contain two or more chemically distinct regions. For example, chimeric oligonucleotides may contain at least one region of modified nucleotides that confer beneficial properties (e.g. increased nuclease resistance, increased uptake into cells), or two or more oligonucleotides of the invention may be joined to form a chimeric oligonucleotide.

The antisense oligonucleotides of the present invention may be ribonucleic or deoxyribonucleic acids and may contain naturally occurring bases including adenine, guanine, cytosine, thymidine and uracil. The oligonucleotides may also contain modified bases such as xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, 2-propyl and other alkyl adenines, 5-halo uracil, 5-halo cytosine, 6-aza uracil, 6-aza cytosine and 6-aza thymine, pseudo uracil, 4-thiouracil, 8-halo adenine, 8-aminoadenine, 8-thiol adenine, 8-thiolalkyl adenines, 8-hydroxyl adenine and other 8-substituted adenines, 8-halo guanines, 8-amino guanine, 8-thiol guanine, 8-thiolalkyl guanines, 8-hydroxyl guanine and other 8-substituted guanines, other aza and deaza uracils, thymidines, cytosines, adenines, or guanines, 5-trifluoromethyl uracil and 5-trifluoro cytosine.

Other antisense oligonucleotides of the invention may contain modified phosphorous, oxygen heteroatoms in the phosphate backbone, short chain alkyl or cycloalkyl intersugar linkages or short chain heteroatomic or heterocyclic intersugar linkages. For example, the antisense oligonucleotides may contain phosphorothioates, phosphotriesters, methyl phosphonates, and phosphorodithioates. In an embodiment of the invention there are phosphorothioate bonds links between the four to six 3'-terminus bases. In another embodiment phosphorothioate bonds link all the nucleotides.

The antisense oligonucleotides of the invention may also comprise nucleotide analogs that may be better suited as therapeutic or experimental reagents. An example of an oligonucleotide analogue is a peptide nucleic acid (PNA) wherein the deoxyribose (or ribose) phosphate backbone in the DNA (or RNA), is replaced with a polyanide backbone which is similar to that found in peptides (J.E. Nielsen, et al Science

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factors such as the nature and amounts of the substance, substrate, and the amount of protein.

- The mRNA for *hfgl2* has multiple AUUUA repeats in the 3' end and this motif binds a set of RNA binding proteins which render the message stable. Removal of the element decreases mRNA stability. Therefore, the invention also contemplates substances which disrupt the AUUUA and RNA binding protein interactions and thereby destabilize the mRNA. The effect of a test substance on the *hfgl2* message may be assayed using conventional methods.

#### (B) Methods of Inducing Fgl2

- In an alternate embodiment, the present invention includes methods of inducing immune coagulation by increasing the activity or expression of Fgl2. Methods that induce immune coagulation may be useful in treating conditions which require an increase in coagulant activity. Such methods can also be used to induce fetal loss.

- Accordingly, the present invention provides a method of inducing immune coagulation comprising administering a nucleic acid sequence encoding Fgl2 or an Fgl2 protein to an animal in need thereof.

- In one embodiment, the invention provides a method of inducing immune coagulation comprising administering (a) a nucleic acid molecule having a sequence shown in Figure 2 or 3 or a fragment thereof or (b) a protein having a sequence shown in Figure 5 or a fragment thereof.

#### (C) Compositions

- The antibodies, antisense oligonucleotides or inhibitors of Fgl2 identified using the methods described herein as well as the Fgl2 protein and nucleic acid sequences, may be incorporated into a pharmaceutical composition containing the substance, alone or together with other active substances.

- In one aspect, the present invention provides a composition for use in inhibiting procoagulant activity in an animal comprising (a) an antibody specific for a Fgl2 protein; (b) antisense nucleic acid molecules complementary to Fgl2; or (c) an inhibitor identified using the method as described above in admixture with a suitable diluent or carrier.

In another aspect, the present invention provides a composition for use in inducing procoagulant activity in an animal comprising a nucleic acid sequence encoding Fgl2 or an Fgl2 protein in admixture with a suitable diluent or carrier.

- Such pharmaceutical compositions can be for oral, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, vaginal, transperitoneal, placental and intracerebral use. They can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

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The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The pharmaceutical compositions can be prepared by per se known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as adjuvants to enhance immune responsiveness.

The antisense nucleic acid molecules of the invention may be used in gene therapy to inhibit immune procoagulant activity. Recombinant molecules comprising an antisense sequence or oligonucleotide fragment thereof, may be directly introduced into cells or tissues *in vivo* using delivery vehicles such as retroviral vectors, adenoviral vectors and DNA virus vectors. They may also be introduced into cells *in vivo* using physical techniques such as microinjection and electroporation or chemical methods such as coprecipitation and incorporation of DNA into liposomes. Recombinant molecules may also be delivered in the form of an aerosol or by lavage. The antisense nucleic acid molecules of the invention may also be applied extracellularly such as by direct injection into cells.

#### (D) Vaccines

The present invention also contemplates a vaccine against a disease involving immune coagulation comprising an amount of an Fgl2 protein or peptide which is effective to induce an immune response against Fgl2. The term "Fgl2 protein or peptide" includes the full length protein (shown in Figure 5) and portions of the protein (peptides) that are useful in inducing an immune response. In one embodiment, the vaccine may comprise a peptide having amino acids 300 to 400 shown in Figure 5, preferably amino acids 364 to 378.

In one embodiment, the present invention provides a vaccine for preventing graft rejection comprising an effective amount of an Fgl2 protein or peptide in admixture with a suitable diluent or carrier. The vaccine may be useful in preventing graft rejection when administered prior to or concurrently with a transplant.

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In another embodiment, the present invention provides a vaccine for preventing fetal loss comprising an effective amount of an Fgl2 protein or peptide in admixture with a suitable diluent or carrier.

The vaccine may be a multivalent vaccine and additionally contain immunogens related to other diseases in a prophylactically or therapeutically effective manner.

The vaccine may also comprise an immunologically acceptable carrier such as aqueous diluents, suspending aids, buffers, excipients, and one or more adjuvants known in the art. Examples of adjuvants include the lipid A portion of gram negative bacteria endotoxin, trehalose dimycolate of mycobacteria, the phospholipid lysolathin, dimethyl dicadecyl ammonium bromide (DDA), linear polyoxypropylene-polyoxyethylene (POP-POE) block polymers and liposomes. The vaccine may also contain cytokines that can enhance the immune response including GM-CSF, IL-2, IL-12, TNF and IFN $\gamma$ . The vaccine may also contain preservatives such as sodium azide, thimerosal, beta propiolactone, and binary ethylenimine.

The vaccines of the invention can be intended for administration to animals, including mammals, avian species, and fish; preferably humans and various other mammals, including bovines, equines, and swine.

The vaccines of the invention may be administered in a convenient manner, such as intravenously, intramuscularly, subcutaneously, intraperitoneally, intranasally or orally. The dosage will depend on the nature of the disease, on the desired effect and on the chosen route of administration, and other factors known to persons skilled in the art.

A vaccine prepared using the methods described herein may be tested in *in vivo* animal systems to confirm their efficacy in the prophylaxis or active immunization and treatment of the relevant disease and to determine appropriate dosages and routes of administration.

The present invention also includes the use of the antibodies that bind the Fgl2 proteins and portions thereof of the invention as a means of passive immunization.

The present invention also includes DNA immunization with an Fgl2 gene or portion thereof. The Fgl2 gene may have the sequence shown in Figure 2 or 3 or SEQ.ID.NO.:1 or 3. A portion of an Fgl2 gene preferably includes a nucleic acid molecule encoding a peptide comprising the amino acid residues at positions 300 to 400 in Figure 5.

## 2. In-vitro Testing and Animal Models

The utility of the inhibitors, antibodies, antisense nucleic acid molecules, Fgl2 protein and nucleic acid molecules and compositions of the invention may be confirmed in *in vitro* systems and animal model systems. For example, proliferation, transcription and/or expression of immune coagulants may be determined in one and two way mixed lymphocyte assays carried out in the presence or absence of antibodies. The effect of a

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substance on procoagulant activity associated with hepatitis may be tested in a murine model of fulminant hepatitis (MacPhee et al., 1985).

Concordant and discordant xenotransplantation transplant models may also be used to confirm the utility of the substances, antibodies, antisense nucleic acid molecules, and compositions of the invention. For example, the following concordant and discordant models may be used:

- rodent model - concordant (Lewis rat to Balb/c mouse)
- rodent model - discordant (guinea pig to rat) (using vascularized heterotropic heart)
- 10 primate model - discordant (guinea pig to rat) (using kidney transplant model)

In concordant and discordant models for testing monoclonal antibodies the following protocol may be used. Recipient animals may receive about 100 µg of purified antibody two days prior to transplant and for 10 to 14 days after. Tissues may be examined for ability of monoclonal antibodies to prevent fibrin disposition, platelet adherence and cellular infiltration. Further testing can be carried out in porcine to primate xenotransplantation using DAF and non-DAF pigs as donors. For these tests animals may be given about 5 mg/kg/animal/day of antibody. For concordant rodent studies animals may receive Neoral (10 mg/kg/i.m.) and/or Cyclophosphamide (40 mg/kg) in addition to the antibody. Control animals will receive an irrelevant antibody of similar isotope. In discordant transplants, in addition to the antibody, some of the animals may receive cobra venom factor and/or Neoral and cyclophosphamide. Pig to primate experiments may be conducted using similar protocols.

The invention also provides methods for examining the function of the *Fgl2* protein encoded by the nucleic acid molecule of the invention. Cells, tissues, and non-human animals lacking in expression or partially lacking in expression of the protein may be developed using recombinant molecules of the invention having specific deletion or insertion mutations in the nucleic acid molecule of the invention. A recombinant molecule may be used to inactivate or alter the endogenous gene by homologous recombination, and thereby create a deficient cell, tissue or animal. Such a mutant cell, tissue or animal may be used to define specific cell populations, developmental patterns and *in vivo* processes, normally dependent on the protein encoded by the nucleic acid molecule of the invention.

To confirm the importance of the *fgl2* protein in transplantation, an *Fgl2* knockout mouse can be prepared. By way of example, a targeted recombination strategy may be used to inactivate the endogenous *fgl2* gene. A gene which introduces stop codons in all reading frames and abolishes the biological activity of the prothrombinase may be inserted into a genomic copy of the fibrinogen like protein. The mutated fragment may be introduced into embryonic stem cells and colonies may be selected for homologous

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- recombination with positive (neomycin)/negative(gancyclovir, thymidine kinase) resistance genes. To establish germ line transmission, two clones carrying the disrupted prothrombinase gene on one allele may be injected into blastocysts of C57/B16 mice and transferred into B6/SJL foster mothers. Chimeras may be mated to C7B1/6 mice and progeny analysed to detect animals homozygous for the mutation (prothrombinase -/-). The effects of the mutation on immune response (allo and xeno transplantation, viral hepatitis) in comparison to non-mutated controls may be determined, and the survival and histologic pattern of disease may be analyzed.

### (3) DIAGNOSTIC APPLICATIONS

- The finding by the present inventor that Fgl2 is a direct prothrombinase involved in immune coagulation allows the detection of conditions involving an increase in Fgl2 prothrombinase.

- Accordingly, the present invention provides a method of detecting a condition associated with immune coagulation comprising assaying a sample for (a) a nucleic acid molecule encoding an Fgl2 protein or a fragment thereof or (b) an Fgl2 protein or a fragment thereof.

#### (i) Nucleic acid molecules

- The nucleic acid molecules encoding Fgl2 or fragments thereof, allow those skilled in the art to construct nucleotide probes for use in the detection of nucleotide sequences encoding fgl2 or fragments thereof in samples, preferably biological samples such as cells, tissues and bodily fluids. The probes can be useful in detecting the presence of a condition associated with immune coagulation or monitoring the progress of such a condition. Accordingly, the present invention provides a method for detecting a nucleic acid molecules encoding Fgl2 comprising contacting the sample with a nucleotide probe capable of hybridizing with the nucleic acid molecule to form a hybridization product, under conditions which permit the formation of the hybridization product, and assaying for the hybridization product.

- Example of probes that may be used in the above method include fragments of the nucleic acid sequences shown in Figure 2 and 3 or SEQ.ID.NO.:1 or 3. A nucleotide probe may be labelled with a detectable substance such as a radioactive label which provides for an adequate signal and has sufficient half-life such as  $^{32}\text{P}$ ,  $^3\text{H}$ ,  $^{14}\text{C}$  or the like. Other detectable substances which may be used include antigens that are recognized by a specific labelled antibody, fluorescent compounds, enzymes, antibodies specific for a labelled antigen, and chemiluminescence. An appropriate label may be selected having regard to the rate of hybridization and binding of the probe to the nucleic acid to be detected and the amount of nucleic acid available for hybridization. Labelled probes may be hybridized to nucleic acids on solid supports such as nitrocellulose filters or nylon membranes as generally described in Sambrook et al, 1989, Molecular Cloning, A Laboratory

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Manual (2nd ed.). The nucleotide probes may be used to detect genes, preferably in human cells, that hybridize to the nucleic acid molecule of the present invention preferably, nucleic acid molecules which hybridize to the nucleic acid molecule of the invention under stringent hybridization conditions as described herein.

- 5 Nucleic acid molecules encoding a Fgl2 protein can be selectively amplified in a sample using the polymerase chain reaction (PCR) methods and cDNA or genomic DNA. It is possible to design synthetic oligonucleotide primers from the nucleotide sequence shown in Figures 2 and Figure 3 for use to PCR. A nucleic acid can be amplified from cDNA or genomic DNA using oligonucleotide primers and standard PCR amplification techniques. The amplified nucleic acid can be cloned into an appropriate vector and characterized by DNA sequence analysis. cDNA may be prepared from mRNA, by isolating total cellular mRNA by a variety of techniques, for example, by using the guanidinium-thiocyanate extraction procedure of Chirgwin et al., *Biochemistry*, 18, 5294-5299 (1979). cDNA is then synthesized from the mRNA using reverse transcriptase (for example, Moloney MLV reverse transcriptase available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase available from Seikagaku America, Inc., St. Petersburg, FL).

(ii) Proteins

- 20 The Fgl2 protein may be detected in a sample using antibodies that bind to the protein as described in detail above. Accordingly, the present invention provides a method for detecting a Fgl2 protein comprising contacting the sample with an antibody that binds to Fgl2 which is capable of being detected after it becomes bound to the Fgl2 in the sample.

- 25 The binding of the antibodies to the Fgl2 protein may be detected using a variety of known techniques including ELISA, radioimmunoassay or histochemical tests. Thus, the antibodies may be used to quantify the amount of the protein in a sample in order to determine its role in particular cellular events or pathological states and to diagnose and treat such pathological states.

- 30 In particular, the polyclonal and monoclonal antibodies against Fgl2 may be used in immuno-histochemical analyses, for example, at the cellular and sub-subcellular level, to detect a protein of the invention, to localise it to particular cells and tissues, and to specific subcellular locations, and to quantitate the level of expression.

- 35 Cytochemical techniques known in the art for localizing antigens using light and electron microscopy may be used to detect a protein of the invention. Generally, an antibody specific for the protein may be labelled with a detectable substance as described herein and the protein may be localised in tissue based upon the presence of the detectable substance.

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Indirect methods may also be employed in which the primary antigen-antibody reaction is amplified by the introduction of a second antibody, having specificity for the antibody reactive against the protein encoded by the nucleic acid molecule of the invention.

#### 5 4. FGI2 GENES AND PROTEINS

As hereinbefore mentioned, the present inventor has cloned and sequenced genomic *hFigl2*. In this regard, the entire genomic sequence as well as the sequence of the promoter region, shown in Figure 8, and the 3' UTR, shown in Figure 4, are included within the scope of the invention.

10 Accordingly, in one embodiment the present invention provides an isolated nucleic acid molecule comprising (a) the sequence shown in Figure 8, where T can also be U; (b) nucleic acid sequences which have substantial sequence identity with (a); and (c) a fragment of (a) or (b).

15 In another embodiment the present invention provides an isolated nucleic acid molecule comprising (a) the sequence shown in Figure 4, where T can also be U; (b) nucleic acid sequences which have substantial sequence identity with (a); and (c) a fragment of (a) or (b).

20 The present invention also includes fragments of the nucleic acid sequences shown in Figure 2 or 3 or SEQ.ID.NO.:1 or 3 which have particular utility in the methods and compositions described above. The fragments generally comprise a nucleic acid sequence having at least 15 bases which will hybridize to the sequences shown in Figures 2 and 3 or SEQ.ID.NO.:1 or 3 under stringent hybridization conditions.

25 Stringent hybridization conditions are those which are stringent enough to provide specificity, reduce the number of mismatches and yet are sufficiently flexible to allow formation of stable hybrids at an acceptable rate. Such conditions are known to those skilled in the art and are described, for example, in Sambrook, et al, (1989, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor). By way of example only, stringent hybridization with short nucleotides may be carried out at 5-10 below the  $T_m$  using high concentrations of probe such as 0.01-1.0 pmole/ml.

30 Fragments of the nucleic acid molecules encoding an immunogenic portion of a human *Figl2* protein are particularly contemplated within the scope of the invention. Preferably, such fragments encode a portion of the human *Figl2* protein which portion binds with an affinity of at least about  $10^6$  l./mole to an antibody raised against human *Figl2*. The present invention in particular contemplates nucleic acids encoding the amino acids at positions 300 to 400, preferably 364 to 378 in the amino acid sequence shown in Figure 5.

35 The invention further includes nucleic acid molecules encoding truncations of the protein encoded by the human *figl2* gene, and analogs and homologs of the protein and

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truncations thereof, as described herein. It will also be appreciated that variant forms of the nucleic acid molecules of the invention which arise by alternative splicing of an mRNA corresponding to a cDNA of the invention are encompassed by the invention. It is also contemplated that nucleic acid molecules of the invention will be prepared having mutations such as insertion or deletion mutations, e.g. nucleic acid molecules encoding analogs of the human Fgl2 protein.

Further, it will be appreciated that the invention includes nucleic acid molecules comprising nucleic acid sequences having substantial sequence identity with the nucleic acid sequences shown in Figures 2, 4 and 8 and fragments thereof having at least 15 bases which will hybridize to these sequences under stringent hybridization conditions. The term "sequences having substantial sequence identity" means those nucleic acid sequences which have slight or inconsequential sequence variations from the sequences disclosed in Figures 2 and 3, i.e. the sequences function in substantially the same manner to produce substantially the same activity as described herein for Fgl2. The variations may be attributable to local mutations or structural modifications. Nucleic acid sequences having substantial identity include nucleic acid sequences having at least 72%, preferably at least 75-95% identity with the nucleic acid sequences as shown in Figure 2 and Figure 3.

Isolated and purified nucleic acid molecules encoding a protein having the activity of human Fgl2 as described herein, and having a sequence which differs from the nucleic acid sequence shown in Figure 2 and Figure 3 due to degeneracy in the genetic code are also within the scope of the invention. Such nucleic acids encode functionally equivalent proteins (e.g., a protein having human Fgl2 prothrombinase activity) but differ in sequence from the sequence of Figure 2 and Figure 3 due to degeneracy in the genetic code.

DNA sequence polymorphisms within the nucleotide sequence of human Fgl2 may result in silent mutations in the DNA which do not affect the encoded amino acid. However, DNA sequence polymorphisms may lead to changes in the amino acid sequences of human Fgl2 within a population. These variations in one or more nucleotides (up to about 3-4% of the nucleotides) of the nucleic acids encoding proteins having the activity of human Fgl2 may exist among individuals within a population due to natural allelic variation. Such nucleotide variations and resulting amino acid polymorphisms are within the scope of the invention.

The nucleic acid molecules of the invention can be used to isolate an Fgl2 from other species. For example, a labelled nucleic acid probe based on all or part of the nucleic acid sequence shown in Figure 2 and 3 can be prepared, and used to screen an appropriate DNA library (e.g. a cDNA or genomic DNA library). Nucleic acids isolated by screening of a cDNA or genomic DNA library can be sequenced by standard techniques.

RNA can be isolated by cloning a cDNA encoding a human Fgl2 protein into an appropriate vector which allows for transcription of the cDNA to produce an RNA

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molecule which encodes a protein which exhibits Fgl2 prothrombinase activity. For example, a cDNA can be cloned downstream of a bacteriophage promoter, (e.g. a T7 promoter) in a vector. cDNA can be transcribed *in vitro* with T7 polymerase, and the resultant RNA can be isolated by standard techniques.

- 5 A nucleic acid molecule of the invention including fragments, may also be chemically synthesized using standard techniques. Various methods of chemically synthesizing polynucleotides are known, including solid-phase synthesis which, like peptide synthesis, has been fully automated in commercially available DNA synthesizers (See e.g., Itakura et al. U.S. Patent No. 4,598,049; Caruthers et al. U.S. Patent No. 4,458,056; and Itakura U.S. Patent Nos. 4,401,796 and 4,373,071).

- 10 Determination of whether a particular nucleic acid molecule encodes a protein having the activity of human Fgl2 can be accomplished by expressing the cDNA in an appropriate host cell by standard techniques, and testing the ability of the expressed protein to exhibit prothrombinase activity as described herein. A cDNA having the biological activity of human Fgl2 so isolated can be sequenced by standard techniques, such as dideoxynucleotide chain termination or Maxam-Gilbert chemical sequencing, to determine the nucleic acid sequence and the predicted amino acid sequence of the encoded protein.

- 20 The initiation codon and untranslated sequences of human Fgl2 may be determined using currently available computer software designed for the purpose, (e.g. PC/Gene (IntelliGenetics Inc., Calif.). The nucleic acid sequence for a 3' untranslated region of *hfgl2* is shown in Figure 4. The intron-exon structure and the transcription regulatory sequences of the gene encoding human Fgl2 may be identified by using a nucleic acid molecule of the invention encoding human Fgl2 to probe a genomic DNA clone library.
- 25 Regulatory elements can be identified using conventional techniques. The function of the elements can be confirmed by using them to express a reporter gene such as the bacterial gene *lacZ* which is operatively linked to the elements. These constructs may be introduced into cultured cells using standard procedures or into non-human transgenic animal models. Such constructs may also be used to identify nuclear proteins interacting with the elements, using
- 30 techniques known in the art.

- In addition to the full length amino acid sequence (Figure 5), the proteins of the present invention include truncations and analogs, and homologs of the protein and truncations thereof as described herein. A truncated Fgl2 protein or fragment of the human Fgl2 protein is a portion of the full-length Fgl2 amino acid sequence having one or more amino acid residues deleted. The deleted amino acid residue(s) may occur anywhere in the polypeptide, including at either the N-terminal or C-terminal end or internally. Fgl2 fragments typically will have a consecutive sequence of at least 10, 15, 20, 25, 30, or 40 amino acid residues that are identical to the sequences of the human Fgl2. The truncations

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or portions of the Fgl2 protein may comprise an antigenic site that is capable of cross-reacting with antibodies raised against the Fgl2 protein whose sequence is shown in Figure 5. Therefore, immunogenic portions or fragments of human Fgl2 proteins are within the scope of the invention (e.g. amino acids 300 to 400 in Figure 5). Preferably the truncated protein or portion of the protein binds with an affinity of at least about  $10^6$  L/mole to an antibody raised against human Fgl2.

At the amino terminal end, the truncated proteins may have an amino group (-NH<sub>2</sub>), a hydrophobic group (for example, carbobenzoxy, dansyl, or T-butyloxycarbonyl), an acetyl group, a 9-fluorenylmethoxy-carbonyl (FMOC) group, or a macromolecule including but not limited to lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates. The truncated proteins may have a carboxyl group, an amide group, a T-butyloxycarbonyl group, or a macromolecule including lipid-fatty acid conjugates, polyethylene glycol, or carbohydrates at the carboxy terminal end.

The proteins of the invention may also include analogs of human Fgl2 as shown in Figure 5 and/or truncations thereof as described herein, containing one or more amino acid substitutions, insertions, and/or deletions. Amino acid substitutions may be of a conserved or non-conserved nature. Conserved amino acid substitutions involve replacing one or more amino acids with amino acids of similar charge, size, and/or hydrophobicity characteristics. When only conserved substitutions are made the resulting analog should be functionally equivalent to human Fgl2 as described herein. Non-conserved substitutions involve replacing one or more amino acids with one or more amino acids which possess dissimilar charge, size, and/or hydrophobicity characteristics.

(One or more amino acid insertions may be introduced into the amino acid sequence as shown in Figure 5. Amino acid insertions may consist of single amino acid residues or sequential amino acids ranging from 2 to 15 amino acids in length. For example, amino acid insertions may be used to destroy the prothrombinase activity of the protein.

Deletions may consist of the removal of one or more amino acids, or discrete portions (e.g. amino acids) from the human Fgl2 amino acid sequence as shown in Figure 5. The deleted amino acids may or may not be contiguous. The lower limit length of the resulting analog with a deletion mutation is about 10 amino acids, preferably 100 amino acids.

The proteins of the invention also include homologs of human Fgl2 as shown in Figure 5 and/or truncations thereof as described herein. Such homologs are proteins whose amino acid sequences are comprised of the amino acid sequences of human Fgl2 regions from other species that hybridize under stringent hybridization conditions (see discussion of stringent hybridization conditions herein) with a probe used to obtain human Fgl2 as shown in Figure 5. It is anticipated that a protein comprising an amino acid

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sequence which is at least 72% preferably 75 to 90% similar, with the amino acid sequence shown in Figure 5 will exhibit prothrombinase activity.

The invention also contemplates isoforms of the human Fgl2 protein of the invention. An isoform contains the same number and kinds of amino acids as the protein of the invention, but the isoform has a different molecular structure. The isoforms contemplated by the present invention are those having the same properties as the protein of the invention as described herein.

The present invention also includes a human Fgl2 protein conjugated with a selected protein, or a selectable marker protein (see below) to produce fusion proteins.

The protein encoded by nucleic acid molecules of the invention, or portion thereof, may be prepared using recombinant DNA methods. Accordingly, the nucleic acid molecules of the present invention or a fragment thereof may be incorporated in a known manner into an appropriate expression vector which ensures good expression of the protein. Possible expression vectors include but are not limited to cosmids, plasmids, or modified viruses, so long as the vector is compatible with the host cell used.

The invention therefore contemplates a recombinant molecule of the invention containing a nucleic acid molecule of the invention, or a fragment thereof, and the necessary elements for the transcription and translation of the inserted sequence. Suitable transcription and translation elements may be derived from a variety of sources, including bacterial, fungal, viral, mammalian, or insect genes. Selection of appropriate transcription and translation elements is dependent on the host cell chosen as discussed below, and may be readily accomplished by one of ordinary skill in the art. Examples of such elements include: a transcriptional promoter and enhancer or RNA polymerase binding sequence, a ribosomal binding sequence, including a translation initiation signal. Additionally, depending on the host cell chosen and the vector employed, other genetic elements, such as an origin of replication, additional DNA restriction sites, enhancers, and sequences conferring inducibility of transcription may be incorporated into the expression vector. It will also be appreciated that the necessary transcription and translation elements may be supplied by the native gene and/or its flanking regions.

The recombinant molecules of the invention may also contain a reporter gene encoding a selectable marker protein which facilitates the selection of host cells transformed or transfected with a recombinant molecule of the invention. Examples of reporter genes are genes encoding a protein such as  $\beta$ -galactosidase (e.g. lac Z), chloramphenicol, acetyl-transferase, firefly luciferase, or an immunoglobulin or portion thereof such as the Fc portion of an immunoglobulin preferably IgG. Transcription of the reporter gene is monitored by changes in the concentration of the reporter protein such as  $\beta$ -galactosidase, chloramphenicol acetyltransferase, or firefly luciferase. This makes it

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possible to visualize and assay for expression of recombinant molecules of the invention and in particular to determine the effect of a mutation on expression and phenotype.

Recombinant molecules can be introduced into host cells via transformation, transfection, infection, electroporation etc. Methods for transforming transfecting, etc. host cells to express foreign DNA are well known in the art (see, e.g., Itakura et al., U.S. Patent No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Corddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference).

Suitable host cells include a wide variety of prokaryotic and eukaryotic host cells, including bacterial, mammalian, yeast or other fungi, viral, plant, or insect cells.

The protein encoded by the nucleic acid molecule of the invention, or portions thereof, may be expressed in non-human transgenic animals such as, mice, rats, rabbits, sheep and pigs (see Hammer et al. (Nature 315:680-683, 1985), Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:4438-4442, 1985), Palmiter and Brinster (Cell 41:343-345, 1985) and U.S. Patent No. 4,736,866).

The proteins of the invention, and portions thereof may also be prepared by chemical synthesis using techniques well known in the chemistry of proteins such as solid phase synthesis (Merrifield, 1964, J. Am. Chem. Assoc. 85:2149-2154) or synthesis in homogenous solution (Houbenweyl, 1987, Methods of Organic Chemistry, ed. E. Wansch, Vol. 15 I and II, Thieme, Stuttgart).

The proteins of the invention may be conjugated with other molecules, such as proteins or polypeptides. This may be accomplished, for example, by the synthesis of N-terminal or C-terminal fusion proteins. Thus, fusion proteins may be prepared by fusing, through recombinant techniques, the N-terminal or C-terminal of the protein, and a selected protein with a desired biological function. The resultant fusion proteins contain the protein or a portion thereof fused to the selected protein.

The following non-limiting examples are illustrative of the present invention:

### EXAMPLES

#### Example 1

##### 1. Cloning The Human Prothrombinase Gene (hfgl2):

##### Methods:

##### 35 a) Screening the PAC library and verifying the clones

Human genomic DNA from the liver was amplified by Polymerase Chain Reaction using primers specific to the human cDNA sequence obtained from GenBank, that corresponds to exon 2 of mouse Ig12 gene; the sense primer CAA AAG AAG CAG TGA GAC

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CTA CA (hufp17) is at position 692, and the antisense primer ITA TCT GGA GTG GTG AAA AAC TT (hufp8) is at position 1133 of the human cDNA. The PAC library, from Genome Systems Inc. (St. Louis, Missouri), was screened using the single amplicon, of about 300 nucleotides in length, that was produced from the above Polymerase Chain Reaction.

- 5 Three clones, namely 6359, 6360, and 6361 were found positive for this screening. The plasmids containing these three clones were purified using the Qiagen maxiprep DNA purification protocol. The quality of the purified DNA and the presence of the inserts were verified by digesting the plasmid with Not 1 restriction enzyme (Canadian Life, Burlington, Canada), and subjecting the samples to Clamped Homogenous Electric Field (CHEF) gel electrophoresis, at 120 angle, 6 Volts, 1-20 seconds ramp interval, 0.5X TBE, and run time of 18 hours.

b) Preparing Sau 3A library

- The clone 6360 was chosen for the rest of the study because in a dot blot analysis it consistently hybridized to sense primer GCA AAC AAT GAA ACA GAG GAA A (hufp1) at position 100 and anti-sense primer at position ATT GCC CTA TTA GAT AAC CAA TAC (hufp2) at position 1400. In order to reduce the DNA into fragments of 5 to 10 kb, which is a convenient size range to work with, the 6360 clone was digested under sub-optimal conditions with the restriction enzyme Sau 3A (Canadian Life, Burlington, Canada). The appropriate digest condition was found by incubating 5 µg of DNA with 1 µl of 2 µl, 0.5 µl, and 0.1 µl of Sau 3A for one hour, at 37°C in a total reaction volume of 20 µl and observing the size range of the DNA fragments on a CHEF gel; the run conditions are 1 to 10 seconds ramp interval, 4.5 volts, 120 angle, 0.5X TBE, and a run time of 16 hours. The 6360 clone was large scale restriction digested by proportionately increasing the amount of DNA, reaction volume, and the amount of enzyme, that is, 10 µg, 40 µl, and 2 µl respectively. The final products of the restriction digest were subjected to CHEF gel electrophoresis at the above conditions. The DNA band corresponding to 6-9kb was excised and fragments were extracted using the Gene Clean DNA purification kit (Bio/Can Scientific, Mississauga, Ontario). The fragments were ligated into the alkaline phosphatase (Pharmacia, Uppsala, Sweden) treated BamHI site of the Bluescript II vector (Stratagene) and transfected into DH10B competent cells by electroporation. This Bluescript II library was screened using the hufp1 and 2 primers. The primers were labeled at the 5 end with gamma P32 by using the enzyme Polynucleotide Kinase (Pharmacia, Uppsala, Sweden); these primers were used to screen the Bluescript library. The clone J14 hybridized to both these primers and was used for the subsequent work.

35 c) Sequencing

The J14 clone was sequenced by Sanger dideoxy chain termination method (Pharmacia, Uppsala, Sweden) and the appropriate primers. The sequence was read from the autoradiograph using the Helixx sequence reading equipment (Helixx Technologies Inc.,

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Scarborough, Ontario). New primers are designed based on the outcomes of manual sequencing and the published cDNA sequence.

#### Results and discussion of cloning hfgl2:

The organization of the J14 clone is summarized in Figure 1. The coding region has been extensively analyzed and compared to the mouse gene. In order to gather insight into the functional properties of this gene, the protein sequence was predicted from the genomic sequence and compared to mouse fgl2 (direct prothrombinase) protein and other relevant coagulation proteases.

#### i) The transcribed region:

A reported cDNA sequence starts 35 bases upstream of the translation start site (Ruegg and Pytela, 1995). The first nucleotide of the reported cDNA is considered the putative transcription start site. In eukaryotes the transcription start site (+1) has a weak consensus of pyC(-1 A+1 NT/Apypp, where adenine at the third position is the transcription start site (Javahery et al., 1994). The cytosine at -1 position is more conserved than the adenine at +1 position (Bucher, P., 1990; Bucher and Trifonov, 1986). In the hfgl2 cDNA, the first nucleotide is a cytosine, and the transcription initiation site does not comply to the above consensus. In the coding region there are long stretches of conserved sequences. In an alignment of exon 1 of fgl2 and hfgl2 there are gaps in the mouse sequence, these gaps correspond to the amino acids that are missing in the mouse protein (Figure 2). Note that in Figure 2, the 5 untranslated regions (UTR) of fgl2 and hfgl2 are not included. Most of the mismatches are at the third nucleotide of the codons, because of codon redundancy, these nucleotide difference do not translate to differences at the amino acid level. The second exon, which corresponds to the carboxyl end of the protein, is more conserved than exon 1 (Figure 3). In Figure 3, exon 2 is included only until the translation stop site because the mouse and the human fgl2 sequences diverge after the translation stop site. The consensus for the 5 splice junction is A/CAG(-1)G(+1)TAAGT where cleavage occurs between -1 and +1 (Breathnach and Chambon, 1981). This splice site consensus is observed in the human fgl2 gene. The 3 splice site consensus is (py)nNpyAG(-1)G(+1) (Breathnach and Chambon, 1981). In the human gene the 3 end sequence of the intron is conserved but not the exon sequence; hfgl2 has a thymidine instead of a guanosine. Except for an additional guanosine at position 14 of 5UTR of the genomic DNA, the coding regions of the genomic and the cDNA are identical until the last 39 nucleotides of the cDNA. A primer designed on this region, hufp15, does not hybridize to the 3 PACs. This region is not likely to be exon III because in mouse fgl2 there is no evidence for the presence of intron 2 and also, this region of hfgl2 does not have the consensus for intron splicing (Koyama et al., 1987). Figure 4 is the incompletely sequenced 3 UTR of hfgl2.

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**(i) Protein Structure:**

The hfg12 protein is 439 amino acids long. The first 204 amino acids are coded by exon 1. The 205th amino acid, a valine, is coded by both exon 1, one nucleotide, and exon 2, two nucleotides. The rest of the 234 amino acids are coded by exon 2. The human protein is 7 amino acids longer than the mouse protein; the extra amino acids are coded by exon 1. Comparing the mouse and the human protein sequence, the carboxyl end is more conserved than the amino terminus (Figure 5). There are five glycosylation sites and every one of them are conserved in the mouse and human proteins (Figure 6). The amino terminus is the most hydrophobic region of the protein. The region between the alanines in positions 12 and 23 is highly hydrophobic. The amino acids between leucine at position 3 and serine at position 11 are moderately hydrophobic. Because of the short hydrophobic stretch of amino acids, it is uncertain whether hfg12 is a transmembrane protein or a secreted protein. For the reasons given below, it is more likely to be a type II ectoprotein than a secreted protein. The transmembrane region tends to conform to a helical structure, which is suggested in Figure 7. Leucine, isoleucine, phenylalanine, and valine are classified as strong  $\beta$  sheet formers, yet in a highly nonpolar environment such as a lipid bilayer, these residues will be forced to assume an alpha helical conformation in order to create a stable, maximally hydrogen bonded structure (Reithmeier and Deber, 1992). Above all, there are no signal peptide cleavage sites; in a signal peptide, cleavage occurs on the carboxyl side of the small aliphatic residues, with the most common cleavage site being alanine (50%), followed by glycine (24%), serine (12%) and cysteine (8%) (Reithmeier and Deber, 1992). It has been proposed that the orientation of membrane proteins is dependent upon differences in the charges of the residues flanking each side of the first hydrophobic segment. Basic residues tend to be found on the cytoplasmic side of the membrane, which is seen in hfg12 (Reithmeier and Deber, 1992).

Based on the catalytic site, proteases are classified as serine, cysteine, aspartate, or metallo proteases (Nduwimana et al., 1995). In their active form, all essential coagulation factors and their regulators are serine proteases; and they belong to family 1 of the clan S<sub>A</sub> (Davie et al., 1991). DFP (Diisopropyl Fluoro Phosphate) inhibition assays imply that fgl2, the mouse direct prothrombinase, is a serine protease (Levy et al., 1983). The predicted amino acid sequence also indicates that hfg12 has a greater potential to be a serine protease instead of a cysteine protease (Tables 1 and 2). In hfg12 there are no serines that are in the same context as the catalytic serine residues of the serine proteases of the coagulation cascade, GDSCG (Barrett and Rawlings, 1995; Rawlings and Barrett, 1994). Both hfg12 and fgl2 could be clan S<sub>E</sub> serine protease, as in Table 2 (Rawlings and Barrett, 1994). The fgl2 protein also has some similarity to clan 1 cysteine protease, as on Table 2.



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After the cysteine at position 212, the rest of the carboxyl end of the protein consists of a domain homologous to the FRBDa (Fibrinogen Related Domain) which is found on a number of different proteins with functional diversity. Some of these proteins are the three chains of fibrinogen, tenascin, fibronin, HPRRP-1, etc (Ruegg and Pytela, 1995; Koyama et al., 1987; Doolittle, R.R., 1984).

## 2. Characterization Of The Promoter Region:

### Method:

The f14 clone contains about 1350 nucleotides upstream of the coding region hfgl2. This entire region was sequenced by Sanger dideoxy chain termination method (Pharmacia, Uppsala, Sweden). The sequence was read from the autoradiograph using Helixx sequence reader (Helixx Technologies Inc., Scarborough, Ontario). Table 3 lists the primers used for sequencing this putative promoter region of hfgl2. The sequence was analyzed using the DNASIS for Windows, sequence analysis software (Hitachi Software Engineering America Ltd., San Bruno, CA) for putative transcription factor binding sites.

### 15 Results and Discussion of Sequence Analysis of hfgl2 Putative Promoter Region:

The promoter region does not have a typical TATA box, TATAAAA, where the adenine in the second and sixth positions, and the thymidine in the third position are more conserved throughout all eukaryotic genes than the rest of the TATA box nucleotides (Bucher, P., 1990; Bucher and Trifonov, 1986). The hfgl2 gene has a TATA like sequence, TATTAAA, about 50 nucleotides upstream of the translation start site (Figures 8 and 9); a typical TATA box is 25 to 30 nucleotides upstream of transcription start site (Bucher, P., 1990; Bucher and Trifonov, 1986). As the TATA and its context are identical in mouse and human fgl2, this region is suggested to be of functional importance.

An AP1 site is located about 20 nucleotides from the TATA box (Figures 8 and 9). The consensus for AP1 motif is TGASTCA, where S is a guanine or a cytosine. Except for the central S, cytosine in humans and guanine in mouse, the AP1 site is identical in mouse and human direct prothrombinase genes. AP1 is composed of dimers of proteins of the Fos and Jun proto-oncogene families. The Jun family members are DNA binding proteins; they bind to the AP1 site as homodimers or as heterodimers with Fos members. Upon activation, Jun gets dephosphorylated at a site proximal to DNA binding domain and acquires its ability to bind DNA (Curran and Franza, 1988; Woodgett et al., 1995). Furthermore, the transactivating domains of Fos and Jun get phosphorylated and are able to interact with the transcription machinery (Woodgett et al., 1995). In certain genes such as tissue factor gene, the AP-1 is required for both constitutive and induced expression (Mackman et al., 1989; Moll et al., 1995).

Interestingly, hfgl2 has 5 Nuclear Factor IL6, (NF IL6), binding sites. The consensus for this transcription factor binding site is T(T/G)NNGNAA(T/G). This region was first identified in the promoter of the IL6 gene; it is located about 350 nucleotides



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upstream of the transcription start site and upregulates the transcription of the IL6 gene (Akira et al., 1995). In hfgl2, the first NF IL6 binding site is located about 300 nucleotides from the translation start site. NF IL6 belongs to C/EBP, CAAT Enhancer Binding Protein, family of transcription factors and any of the C/EBPs can bind to the above consensus sequence (Wedel and Ziegler-Heitbrock, 1995). The carboxyl end of these proteins are conserved and contain the DNA binding basic region and dimerizing leucine zipper region. The amino terminus contain the transactivating domain (Wedel and Ziegler-Heitbrock, 1995). C/EBP $\alpha$  is responsible for transcription of adipocyte specific genes and constitutive expression of liver specific genes such as albumin and transferrin. C/EBP $\beta$  and  $\delta$  play a role in the induction of acute phase response genes of the liver and cytokine genes of macrophages (Akira et al., 1992; Wedel and Ziegler-Heitbrock, 1995; Akira and Kishimoto, 1992). C/EBP $\beta$  or NF IL6 mRNA level is rapidly induced in the macrophages as a result of cytokine induction. Also, the level of C/EBP $\alpha$  and  $\beta$  seem to be inversely related (Akira et al., 1992; Akira and Kishimoto, 1992). Hence, the ratio of the appropriate C/EBP's may influence the expression of hfgl2. In certain hemophiliac B patients mutation is found in the CCAAT box of factor IX gene which indicates the importance of this cis element (Peterson et al., 1990).

TCF-1 (T Cell Factor 1) binds to the A/T A/T CAAAG motif. The expression of this transcription factor appears to be completely restricted to the T cell lineage and is confined to the nucleus (Castrop et al., 1995; Verbeek et al., 1995). The DNASIS software package has selected 10 TCF 1 binding sites in the promoter region of hfgl2 gene. The presence of TCF 1 binding motifs could be responsible for the reported constitutive expression of this gene in T cells (Ruegg and Pytel, 1995; Koyama et al., 1987).

Members of the ets 1 proto-oncogene family binds to the PEA3 domain, Polyomavirus Enhancer Activator 3, which has a consensus sequence AGGAAG (Xin et al., 1992; Wasyluk et al., 1990). There is a PEA3 site about 1200 bp from the translation start site (Figures 8 and 9). There is evidence for the cooperative interaction between AP1 and PEA3 (Wasyluk et al., 1990). Some of the promoters with PEA3 motifs can be grouped according to the type of inducers which activate their transcription i) acute phase response ii) gamma interferon and iii) mitogens and oncogenes (Xin et al., 1992). As fgl2 gene expression is induced by viral infection, hfgl2 gene product could also be an acute phase response protein. The presence of many putative bHLH (Basic Helix Loop Helix) domains, with the consensus of CAXXTG, may imply that this gene is under the influence of many DNA binding transcription activators (Murre et al., 1994). These transcription activator proteins contain a region of mainly basic residues that allows the helix-loop-helix proteins to bind DNA. The second region characterized by mainly hydrophobic residues, the HLH domain, allows these proteins to interact and form either homo or hetero dimers (Murre et al., 1994).





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### 3. Sizing the hfgl2 Intron by PCR:

#### Method:

Intron 1 was amplified by PCR using the primer combinations hufip3 with 6, and hufip 5 with 6 (Table 3). As templates for amplification J14, 6359, 6360, and 6361 clones were used. As mentioned above, 6359, 6360, and 6361 are the PAC clones that contain hfgl2, and J14 is a subclone of 6360 in Bluescript II vector (Stratagene). The reactions were performed in a 50 µl total volume containing 25p moles of each primers, 200 µM each dNTP, 1X PCR, 3 or 5mM MgCl<sub>2</sub>, and 2.5 U of Taq Polymerase (Canadian Life, Burlington, Canada). The reaction was subjected to denaturing at 95°C for 5 minutes, followed by 30 cycles of 1 minute denaturing at 95°C, 1 minute annealing at 60°C, and 2 minute extension at 72°C. The PCR products were electrophoresed in 0.8% agarose, 0.5X TBE, and 0.5 µg/ml ethidium bromide.

#### Results and Discussion of Sizing Hfgl2 Intron:

A 2800bp amplicon was synthesized when the primer set hufip 3 and 6 was used. When hufip 5 and 6 were used the size of the amplicon was 2400 bp. Subtracting the exonic region, intron 1 is about 2200 bp, which is also observed in mouse (Koyama et al., 1987). As mentioned earlier this intron of human fgl2 has the consensus for intron splicing. The consensus for the 5 splice junction is A/CAG(-1)G(+1)TAAGT where cleavage occurs between -1 and +1 (Breathnach and Chambon, 1981). This splice site consensus is observed in human fgl2 gene. The 3 splice site consensus is (py)nNpyAG(-1)G(+1) (Breathnach and Chambon, 1981). In the human gene the 3' end sequence of the intron is conserved but not the exonic sequence; hfgl2 has a thymidine instead of a guanosine.

### 4. RT-PCR of hfgl2 from human small intestine total RNA:

#### Method:

In order to synthesize the first strand cDNA from human small intestine total RNA 4 µg of RNA (Bio/Can Scientific, Mississauga, Canada), 2 µl of Random hexamer at stock concentration of 1 µg/µl (Pharmacia, Uppsala, Sweden), 20U of RNase Inhibitor (Pharmacia, Uppsala, Sweden), and DEPC treated water to bring the volume to 12 µl, were added together and incubated at 65°C for 5 minutes and quickly chilled on ice. Then 1mM each dNTP (1 µl of 20mM stock), 10mM DTT (2 µl of 0.1M DTT), 4 µl of 5X 1st strand buffer (Canadian Life, Burlington, Canada), and 200U MMLV-RTase buffer (Canadian Life, Burlington, Canada) were incubated at 37°C for 1.5 hours. The reaction mix was heated at 95°C for 5 minutes and diluted 5 fold with 80 µl of DEPC treated water.

Polymerase Chain Reaction was performed in a 50 µl reaction volume, with 10 µl of the above RT mix, 2.5 µl of 10pmol/µl of sense and anti-sense primers, 0.2mM dNTP, 1X Taq buffer, 3mM MgCl<sub>2</sub>, and 5U of Taq Polymerase (Canadian Life, Burlington, Canada). The primer sets used for the PCR reaction were hufip1 and 2, hufip1 and 6, hufip5 and 2, hufip13 and 26, hufip13 and 28, hufip15 and 26, hufip15 and 28, and hufip11 and 28.



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The reaction conditions were 95°C for 5 minutes, and 30 cycles of 95°C for 1 minute, 47 or 57°C (depending on the primer set) for 1 minute, and 72°C for 2 minutes. The PCR products were electrophoresed in 0.8% agarose, 0.5X TBE, and 0.5 µg/ml of ethidium bromide.

Ruegg and Pytela had reported the cloning of cDNA, from human small intestine, which is homologous to the gene coding for mouse fibrinogen like protein (Ruegg and Pytela, 1995). The RT-PCR results shows that there is indeed hfgl2 expressed constitutively in human small intestine. The primers hufp26 and 28 are located downstream of primers hufp13 and 15; and all 4 primers are located in the 3'UTR. As the primer combinations hufp13 and 26, and hufp13 and 28 produce amplicons, it shows that the 3' end of the J14 clone is indeed part of an exon. As hufp15 does not synthesize an amplicon with antisense primers, the last 39 nucleotides reported by Ruegg and Pytela must be a cloning artifact.

## 5 Methods

### Results and discussion of restriction mapping:

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restriction map of the PAC clones. The presence of a *Sma*I site within the *hfgl2* gene hastened the process of mapping *hfgl2* within the PAC clones. The approximate sizes of the 3 PAC clones, 6359, 6360, and 6361 are 159 kb, 139 kb, and 116 kb, respectively. The most accurately restriction mapped clone is 6360. The presence of a large number of *Sal*I and *Sma*I sites has limited the accuracy of mapping the clones 6359 and 6361. Figure 11 shows that the orientation of the insert in 6359 is opposite to that of 6360 and 6361.

#### Transient expression of *hfgl2*:

The protein sequence homology between *fgl2* and *hfgl2* suggests that *hfgl2* also codes for a direct prothrombinase. A prototype has been developed to synthesize the cDNA for *hfgl2* that can be used for expression studies. Human small intestine total RNA (Bio/Can Scientific, Mississauga, Canada) is being used as the template for first strand synthesis and random hexamers (Pharmacia, Uppsala, Sweden) are used as primers. For the PCR reaction *hufp29* and *30* are used as primers. The PCR product will be cloned into the vector PCR2.1 (Invitrogen, San Diego, CA). The insert will be rescued from the vector using the restriction enzyme *Eco*RI. This insert, containing the coding region of *hfgl2*, will be cloned into the *Eco*RI site of the vector pcDNA 3.1-(his,myc) (Invitrogen, San Diego, CA). In order to increase the transient transfection efficiency, the vector will be linearized and transfected into COS cell, using lipofectin. The cells will be initially screened using the standard procoagulant assay. Then coagulation assays will be performed in factor deficient plasma to see if they carry the potential to exhibit direct prothrombinase activity. Finally, prothrombin cleaving assays will be performed, in presence of anti tissue factor and anti factor X, by monitoring the cleavage of radio active iodine labelled prothrombin.

#### Northern Blot of human small intestine total RNA:

25                    25 µg of human small intestine total RNA (Bio/Can Scientific, Mississauga, Canada) is run on an agarose gel, transferred to nylon membrane, and probed with exon 1 of *hfgl2* and *GABDH*, separately. This assay is performed to identify the size of *hfgl2* mRNA, and also to detect whether there are more than one species of mRNA for this gene.

#### 30    Example 2

#### PREVENTION OF GRAFT REJECTION BY ANTIBODIES TO FGL2

##### (a) Allograft

In order to study the ability of monoclonal antibodies to rodent *fgl2* to prevent allograft rejection, heterotopic auxiliary small intestinal transplants were undertaken using intestines from donor Lewis Brown Norway F1 (LBNF1) and recipient Lewis rats. The procedures followed for the operation have been described previously (Effects of Cyclosporine and Cyclosporine Metabolites in Experimental Small Intestinal



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Transplantation, P.C.W. Kim, Z. Cohen, P.Y. Wong, E. Cole, J. Cullen, K. Skorecki et. al, Transplantation 1990;49:1043-1050).

Briefly, adult Lewis (LEW) and Lewis x Brown Norway F1 hybrids (LBNF1) female rats weighing 200-250 grams were utilized in all experiments. For 24 hours prior to surgery, animals were starved and donors were gavaged with neomycin 60 mg/kg and erythromycin 40 gm/kg 24 and 12 hours respectively prior to the operation. Heterotopic auxiliary small intestinal transplantation was carried out in a sterile environment with a modification of the procedure described by Monchik and Russel (Monchik, G.), Russel, P. Transplantation of Small Bowel in the Rat: Technical and Immunological Considerations, Surgery 1971;70:633).

**Rats were divided into 4 groups:**

**Group I**      **n=5; received no treatment**

**Group B** n=5; received 7.5 mg/kg of CsA on day 1

Group III received 1 mg/kg of monoclonal antibody to Ig12

15 Group IV received 1 subcutaneous injection of CsA (7.5 mg/kg) and daily injections of monoclonal antibody to IgJ2 (1 mg/kg for 10 days)

The animals were carefully monitored for progress twice daily and at time of demise, tissues (intestine) were removed and sent for routine histology and immunofluorescence for presence of fibrin deposits. At 28 days, surviving animals were sacrificed and allografts analyzed for histology and for presence of fibrin deposits.

Control animals which received no treatment all died within 4 days of transplant and all allografts showed severe necrosis with fibrin deposition. Sixty percent (60%) of animals that received a single injection of cyclosporin A died on day 4 and by day 14 only 1 animal (20%) remained alive. Analysis of tissues from all animals showed marked to severe necrosis of the allografts. Animals which received daily injection of monoclonal antibodies to fgl2 had increased survival and by day 14, 60% (3/5) of these animals were alive (Figure 12). Analysis of allografts showed mild to moderate rejection with no evidence of fibrin deposits. Finally, all animals (5/5) which received a single injection of cyclosporin A and 10 days of monoclonal antibody to fgl2 survived and analysis of their grafts on day 28 showed normal histology.

(b) Xenograft

To study the ability of antibody to fg12 prothrombinase to protect against hyperacute xenograft rejection, livers from Wister rats were perfused with blood from guinea pigs in an isolated liver perfusion apparatus in the presence or absence of 100 mg of antibody to fg12. Livers were removed aseptically from three groups of Wister inbred rats. The livers were then perfused with oxygenated blood from guinea pigs to which was added either 100 mg of normal mouse IgG1 (Group 1); 100 mg of normal rat blood (Group 2); or 100 mg of antibody to fg12 prothrombinase for 120 minutes in an isolated perfusion chamber.



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Livers perfused with normal mouse IgG1 and albumin became dark, stiff with area of fibrinoid necrosis within 10 minutes and by 30 minutes blood could not be perfused into these livers. In contrast, livers perfused with blood to which antibody to fg12 had been added appeared morphologically normal, perfusion pressures remained normal and perfusion was continued for the full 120 minutes after which the livers were harvested and examined by routine histology and electron microscopy. In the livers from groups 1 and 2 serum alanine transaminase (ALT) levels rose precipitously from a baseline of 45 IU/L to 12,400 IU/L at 20 minutes increasing to 18,700 IU/L at 30 minutes when the livers were removed from the perfusion device. In contrast serum ALT levels in livers from group 3 remained at near normal ALT 55 IU/L at 20 minutes; 68 IU/L at 90 minutes and 90 IU/L at 120 minutes. Histology from the livers from groups 1 and 2 showed marked areas of necrosis and hemorrhage with dense intrasinusoidal fibrin deposits. Electron micrographs showed platelet adherence, fibrin deposits and endothelial cell destruction. In contrast, liver architecture appeared near normal in livers from group 3 with only small amount of platelet and fibrin deposits.

These results demonstrate that antibody to fg12 prothrombinase can prevent hyperacute xeno graft rejection.

### Example 3

#### Induction of Fg12 Promoter by XenoSera

### MATERIALS AND METHODS:

#### Vector Constructs

Restriction enzymes used to create promoter constructs were obtained from GIBCO BRL, Life Technologies, Grand Island, N.Y., USA. All plasmids were purified using Qiagen Maxiprep kits, and grown in DH5 E. coli bacteria (GIBCO BRL).

DNA from -3.5kb/+9bp and -1.3kb/+9bp fg1-2 promoter region pGL2-Basic luciferase constructs (pL-3500, pL-1300) was obtained from clones previously constructed in Dr. Levy's lab (unpublished data). Additional 5' truncation series plasmids and the 3' pL3'274 luciferase vector were constructed first using PCR, followed by cloning into a PCR2.1 plasmid (Invitrogen). Specific portions of the pL-3500 clone were amplified at 35 cycles performed at 95 C for 1 min, 58 C for 1 min, 72 C for 2 min. The downstream 3' reverse primer, present in pGL2-Basic, was fixed for all 5' truncations and was 5'-GAA ATA CAA AAA CCC CAG AAG G-3' (Promega). The upstream primer used to construct pL-995 was 5'TCT TGG GAA ATC TCG TTA GAG-3. The upstream primer for pL-681 was 5'-GAG CTG AGT GAT GGG GAA GGA-3'. The upstream primer for pL-294 was 5'-GGG CAC TCG TAT TAC AAC TGT-3', and the 5' primer for pL-119 was 5'-CTC CTC CAG TGT GGC CTC TGA-3'. The fixed 5' forward primer for the 3' truncation was 5'-GGA TAA GGA GGG CAG GGT GAA-3'. The downstream antisense primer for pL3'274 was 5'-ACA GTT GTA ATA CCA GTG CCC-3'. Following PCR, PCR products were ligated and cloned into the PCR2.1 vector. PCR2.1



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clones were sequenced to check for orientation, and DNA was obtained from desired clones. For the 5' truncations, the PCR2.1 clones were digested with KpnI and SalI, and then ligated and cloned into the pGL2-Basic luciferase vector (Promega) cut with KpnI and XhoI. Each final construct was checked with a specific diagnostic digestion before maxi-preps of DNA were made. For pL3274, PCR2.1 clones were digested with EcoRV and HindIII, and then ligated and cloned into pGL2-Basic cut with SmaI and HindIII. A summary of the different constructs produced is shown in Figure 13.

#### Cell Culture and Sera

SVBC4-10 cells (SV 40 transformed axillary lymph node, vascular endothelial cells from C3H/HeJ adult mice) were purchased from American Type Culture Collection (ATCC), MD, USA. The cell cultures were carried, and subcultured as indicated by ATCC, and in 60mm plates (Corning). Cell cultures were kept at 37°C, 5% CO<sub>2</sub>, and for no longer than sixteen passages.

Porcine serum, rat serum, and fetal bovine serum (FBS) were obtained from GIBCO BRL. Human serum was generated from human plasma (kind gift of Dr. Levy); blood samples were allowed to clot at room temperature for 30 min, and the serum (action was removed after 15 min of 2900 RPM centrifugation. Autologous C3H serum was purchased from The Jackson Laboratories, Bar Harbor, Maine, USA. All sera were heat inactivated at 56°C for 45 minutes, aliquotted, and stored at -20°C.

#### DNA Transfections

All transfections were carried out using Lipofectamine (GIBCO BRL). Prior to transfection, 1-3 X 10<sup>5</sup> SVEC4-10 cells were seeded per well into six well (35mm) plates in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) (GIBCO BRL) containing 10% FBS and 1% Penicillin-Streptomycin (GIBCO BRL). The cells were incubated for 18-24 hrs until they were 70-80% confluent, and then transfected. For each well transfected, 2µl of lipofectamine was diluted into 100µl DMEM. This solution was then added to a solution containing 0.5µg of pGL2-Luciferase vector construct and 0.25µg of pRSV-β-Gal vector (Promega) diluted into 100µl of DMEM. In general, solutions for four wells were made at once. The lipofectamine/DNA solution was then vortexed gently, centrifuged for 5 sec at 1500 RPM and allowed to equilibrate for 30 minutes. During this time, the SVEC4-10 cells were washed twice with DMEM. 1 ml of liposomes in DMEM were then added to each well. The transfected cells were incubated at 37°C for 5-6 hrs, after which the transfection medium was replaced with a fresh 2 ml of DMEM. In total, the cells were serum starved 15-20 hrs in DMEM before inducing for 8-10 hrs with various xenosera and autologous serum at different concentrations in DMEM. Thus, cells which did not receive serum were starved for a total of 23-30 hrs. Each experimental well was done in duplicate.



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### Transfection Assays (Luciferase and $\beta$ -Gal Assays)

**Protein Extraction:** Following serum stimulation, the cells were harvested for protein extraction. The cells were first washed once with 2 ml of PBS (GIBCO BRL) and then lysed with 200ul of reporter lysis buffer (Analytical Luminescence Laboratory, MI, USA).

5 Culture plates were kept on ice, and the cells were scraped and collected into 1.5 ml Eppendorf tubes. These extracts were stored at -70 C. On the day of the transfection assays, cell lysates underwent three freeze and thaw cycles from liquid Nitrogen to 37 C to help release intracellular protein. The protein extracts were then centrifuged for 6 min at 14000 rpm and 4 C to pellet cellular debris. The supernatants containing fl-galactosidase and luciferase were kept on ice until use in the transfection assays.

**$\beta$ -Galactosidase ( $\beta$ -gal) Assay:** For each sample assayed, 3 $\mu$ l of 100X Mg solution (0.1M MgCl<sub>2</sub>, 4.5M  $\beta$ -mercaptoethanol) was mixed with 33 $\mu$ l of 2X O-nitrophenyl  $\beta$ -D-Galactopyranoside (Sigma Chemical Co., St. Louis, Mo., USA), 30 $\mu$ l of cell extract and 0.1M sodium phosphate (41% w/v 0.2M Na<sub>2</sub>HPO<sub>4</sub> 2H<sub>2</sub>O, 9% w/v 0.2M NaH<sub>2</sub>PO<sub>4</sub> 2H<sub>2</sub>O in 50% w/v H<sub>2</sub>O) in a 96 well microtiter plate (Costar). The reaction was incubated at 37 C for 30 min, and the optical density was read at 414 nm using a microtiter spectrophotometer. Background  $\beta$ -gal was determined using a lysis buffer control and was subtracted from the other samples. The  $\beta$ -gal assay was used to standardize for transfection efficiency.

**Luciferase Assay:** Cell extracts were assayed for luciferase activity using a MonoLight 2010C luminometer (Analytical Luminescence Laboratory, MI, USA). The luciferase reagents were allowed to thaw to room temperature from -20 C. Then 30µl of protein extract was added to 20µl of 1X Coenzyme A (Sigma) in a 10ml luminometer cuvette (Analytical Luminescence Laboratory, MI, USA) which was loaded into the luminometer. 100µl of 1mM D-luciferin (Analytical Luminescence Laboratory, MI, USA) and 100µl of luciferase lysis buffer (30mM Tricine, 3mM ATP, 15mM MgSO<sub>4</sub>, 10mM DTT) are then injected automatically by the luminometer. Light released was measured over a 10 second period. The pGL2-Enhancer vector (Promega) was used as a positive control and the pGL2-Basic vector was used as a negative control. Output data is expressed in raw luciferase units (RLU). Normalized Luciferase data was obtained by dividing the RLU by the  $\beta$ -gal absorbance and then subtracting off the pGL2-Basic net background luminescence.

### Statistical Analysis:

Quantitative data were expressed as means  $\pm$  standard deviations. Statistical analysis was carried out using the student's t-test, and a P value of less than 0.02 was considered statistically significant.



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**RESULTS****Fgl-2 Promoter Activity is Induced by Xenoseraum but only Insignificantly in Autologous Serum in Endothelial Cells.**

SVBC4-10 is a murine endothelial cell line derived from C3H/HeJ mice.

- 5 These cells were chosen to study fgl-2 promoter responses since they have been shown to respond like normal endothelial cells to different cytokines including various interleukins, and TNF- $\gamma$  (J. Immunol. 144: 521); IFN-gamma induces MHC class II in a time course identical to normal endothelial cells (J. Immunol. 144: 521). Furthermore, endothelial cells are predicted to be a barrier to successful xenotransplantation and are actively involved in
- 10 xenograft rejection, so that analysis of fgl-2 transcription in these cells may represent an in vitro model of a xenograft system.

- To determine transcriptional activity of the fgl-2 promoter in response to xenoseraum versus autologous serum, pL-1300 was transfected in SVBC4-10 cells, following which, they were incubated with either 20% xenoseraum, 20% autologous serum, or no serum.
- 15 Different xenoseraum used for induction included FBS, porcine serum, rat serum, and human serum. Autologous serum was from C3H/HeJ mice. Serum-free conditions contained only DMEM. The relative luciferase activity was calculated for each different serum source used, and is expressed in Figure 14 as a percent relative to serum free conditions. Luciferase activity was normalized for the amount of DNA uptake using a  $\beta$ -Galactosidase construct
- 20 as an internal control. Each transfection was done using pL-1300 in duplicate and at least three times. Shown are the mean values and standard deviations for induction with 20% xenoseraum (FBS, pig, rat, human) and 20% autologous C3H serum. FBS, porcine serum, and rat serum all induced luciferase expression on average about 4 times higher than in serum free conditions. 20% human serum induced fgl-2 promoter transcriptional activity by an
- 25 average of  $2.6 \pm 0.3$  fold. Autologous C3H serum induced only a small increase in fgl-2 promoter activity, and was statistically less than human serum induction ( $P < 0.02$ ).

- Dose response curves for xenoseraum versus autologous serum were constructed. In these experiments, SVBC4-10 cells were transfected with pL-1300, and later induced in the presence of 0.1%, 1%, 3%, 5%, 10%, 20%, 50%, and 100% FBS or porcine serum. Again,
- 30 luciferase activity was expressed as a percent relative to serum free conditions. The dose response curves for FBS, porcine serum and C3H serum are shown in Figure 15. Both FBS and porcine serum induced luciferase activity in a dose dependent manner to a peak of at least 400% of serum free conditions. Autologous C3H serum failed to induce transcription from the fgl-2 promoter at all doses tested. It is also interesting to point out that the luciferase
- 35 activity consistently fell in response to incubation with 100% porcine or FBS to levels comparable to 3% xenoseraum. This drop in promoter activity may indicate some degree of toxicity associated with high serum levels.





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Fetal Bovine Serum Induction is localized to the first 119 bp upstream of the transcription initiation site.

To map the position in the fgl-2 promoter region which responds to xenoserum, plasmids with sequential 5' truncations of the promoter were constructed (see Figure 13). Each construct was transfected into SVEC4-10 cells which were either serum induced with 20% FBS or not induced (DMEM only). The fold increase over the serum free conditions for each construct was then calculated and expressed as a percentage increase. The results are summarized in Figure 16. pL-3500, containing 3.5kbp upstream of the transcription initiation site, was inducible to about 215% (a 2.15 fold induction) of the corresponding non-induced samples. Deletion of the promoter region between -3500 and -1300 led to an almost 2 fold increase in luciferase induction, suggesting that this region might bind to regulatory factors which inhibit xenoserum induced transcription. Constructs with sequential 5' truncations of the fgl-2 promoter region (pL-1300, pL-986, pL-681, pL-294, pL-119) down to nucleotide -119 all were induced to maximal levels (about 350% of non-induced samples) by FBS. Thus, only the first 119 bp upstream of the transcription initiation site of fgl-2 were necessary to maintain optimal induction of fgl-2 transcription by FBS. This observation indicates that there are important DNA sequence elements in this region which are responsible for fgl-2 promoter induction by xenoserum. Most notably, this region of the promoter contains a predicted TATA box at -32, an AP-1 binding site (5'-TGAGTCAG-3') at -51, and an SP1 binding site (5'-CCGCCC-3') at -79 (see Figure 17E). When the first 274 bp upstream of the transcription initiation site were deleted (pL-274), no induction of luciferase activity by FBS was obtained. This plasmid reinforces the importance of the proximal promoter region in FBS induction.

#### DISCUSSION

The above results suggest that a 1.3 kb portion of the fgl-2 promoter region is selectively induced in ECs by xenoserum in a dose dependent manner after 8-10 hours of induction (Figure 14, Figure 15). The selective xenoserum induction observed in this study may be as a result of a number of possibilities. One possibility is that the selective induction is mediated through enoactive natural antibodies (XNAs). According to this view, the autologous C3H serum would not significantly induce fgl-2 promoter activity because it lacks XNAs capable of binding to its EC surface. Insignificant XNA binding would lead to little or no stimulus delivered into the cell to activate fgl-2 transcription. All four xenosera (FBS, pig, rat, human) are known to contain XNAs to various epitopes, some unidentified, on murine ECs which might mediate the fgl-2 promoter activation seen in this study. It is interesting to point out that the most phylogenetically distant xenoserum used, and the only one which contains XNA to Gal 1-3Gal (the human serum), yielded the lowest transcriptional induction from the fgl-2 promoter. This suggests that fgl-2 promoter induction by xenoserum was probably not caused by XNAs to Gal 1-3Gal in



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the absence of complement. It may still be interesting to test for fgl-2 promoter induction at various time points using human serum that is not heat inactivated or human serum to which complement components are added, so that the XNAs to Gal 1-3Gal may fix complement on the murine ECs.

Another possibility which may help explain the selective inducibility of pL-1300 in ECs by xenoserum is the presence of proinflammatory cytokines. At the site of a vascular xenograft rejection, there is a high level of proinflammatory cytokines and soluble factors produced which act on ECs to help mediate the pathology. Some of these include C5a, IL-1, IL-1 $\beta$ , IL-6, IL-7, IL-12, IFN- $\gamma$ , and TNF. (Pfaff, J. L. 1996; Parker, W. et al. 1996; Bach, F.H. et al., 1996). Cytokines in the xenoserum used may have contributed to fgl-2 promoter induction. In favour of this hypothesis is the recent observation that the fgl-2 gene is known to be induced by IFN- $\gamma$  in monocytes (Lafuse, W.P., et al. 1995). Our lab has also shown that both IL-1 and TNF- $\alpha$  can induce ECs to transcribe fgl-2 (Parr, R.L. et al. 1995). Cytokine action may also account for the lower luciferase activity obtained using human serum for induction. It has been shown that certain cytokines do not function across distant species barriers. For instance, human IL-1 $\beta$  and IFN- $\gamma$  do not stimulate porcine ECs (Bach, F.H. et al. 1996). Cytokines in high doses are also known to exhibit toxicity to cells, which, in response, will try to decrease their signal transduction. This idea might explain the down regulation of fgl-2 promoter activity seen in 100% xenoserum from the dose response curve (Figure 15).

The fgl-2 promoter induction by xenoserum (FBS) was mapped to the first 119 bp upstream of the transcription initiation site (Figure 16). This region is rich in consensus sequences that bind regulatory factors and includes an AP-1 binding site at -51 and an Sp1 binding site at -79 (Figure 17). Both AP-1 and Sp1 have been shown to be important in high level serum induction of tissue factor, an important procoagulant molecule expressed in xenograft rejection (Mackman, N. et al. 1990). AP-1 sites, and AP-1-like sites, have also been shown to be important agonist response elements. For example, there is an AP-1-like binding site within the human IL 2 gene which responds to IL-1 stimulation (Muegge, K. et al. 1989). Thus, these sites may play a role in fgl-2 promoter induction by various cytokines present at the site of xenograft rejection.

#### **Example 4**

##### **Monoclonal Antibody To fgl2 As Treatment For Recurrent, Unexplained Fetal Loss**

Studies were undertaken to study the potential usefulness of monoclonal antibodies to the prothrombinase (fgl2) in the treatment of stress triggered fetal loss. Stressing animals has been shown to result in diminished fertility, mating behaviour, ovulation, implantation, fetal growth and lactation (P. Arek, E.S. Motali, J. Manuel, G. Chaquat and D.A. Clark, Stress Triggered Abortion Inhibition of Protective Suppression



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and Promotion of Tumor Necrosis Alpha (TNF- $\alpha$ ) Releases a Mechanism Triggering Receptors in Mice, *American Journal of Reproductive Immunology* 1995;33:74-80).

Exposure to mice to crowded housing conditions, daily handling, forced swimming, loud noise, heat, bright light and physical restraint have been shown to have a deleterious effect on pregnancy outcome in rodents. The type of stress and its effect appears strain dependent. In this CBA/J x DBA/2J matings, ultrasonic sound has been shown to elevate the fetal loss rate. On examination of uteri, it has been shown that a strong pathogenic feature of induced fetal loss is the presence of fibrin deposits.

Therefore, studies were undertaken to determine the efficacy of the  
 11 monoclonal antibody to IgG2 and its ability to prevent fetal resorption and fetal loss.

The methodology has been well defined. Briefly, after overnight cohabitation of 6-8 week old females (CBA/J) to (DBA/2J) males, the females with vaginal plugs were segregated and assigned to receive no treatment or 10 ug of monoclonal antibody to Ig2 IV daily for 10 days. Both groups of animals (n=10 per group) were subjected to stress which consisted of exposure to ultrasonic sound stress for a battery powered rodent repellant device. On day 13.5 of pregnancy, the females were euthanized by cervical dislocation, uteri were removed and opened and examined for the total number of implantations in a number of resorbing sites recorded.

The results are shown in Figure 18. Animals which had not been treated had greater than 70% resorbing sites with less than 30% implantations. In contrast, the animals that had received daily injections of the monoclonal antibodies had less than 10% resorbing sites with 90% implantation. This reduction in fetal loss rate was statistically significant at  $p < 0.001$ . Furthermore, on analysis of uteri in the non-treated animals, there were dense fibrin deposits where these were not detected in animals that had been treated with the monoclonal antibodies.

### Example 5

### Monoclonal Antibodies To Fc $\gamma$ 2 As Treatment For Spontaneous Retal Loss And Fetal Loss Induced By TNF- $\alpha$ And $\gamma$ -INTERFERON

30 Studies were done to investigate the potential usefulness of monoclonal antibody to fgl2 prothrombinase in reducing the risk of spontaneous fetal loss and fetal loss induced by TNF- $\alpha$  and  $\gamma$ -interferon in DBA/2-mated CBA/J mice.

To study of the role of monoclonal antibody to fgl2 prothrombinase in fetal loss, the roles of TNF- $\alpha$  and  $\gamma$ -interferon, NK cells, and macrophages in causing fetal loss were directly tested using in vivo cell depletion techniques and mice deficient in the response to interferon.

Cytokines TNF- $\alpha$  and  $\gamma$ -interferon play an important role in fetal loss as their administration increases the fetal loss risk and specific antagonists decrease the fetal loss risk (Chaouat, G., et al., J. Reprod. Fert. 89:447 (1990); Arch, P.C., et al., Amer. J.



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Reprod. Immunol. 37:262 (1997); Chauvat, C. et al., J. Immunol. 154:4261 (1995); Gendron, R.L. et al., J. Reprod. Fertil. 90:447 (1990)).

In the experiments that follow, the inventor demonstrates that in induced fetal loss the fetal loss is caused by ischemia due to activation of vascular endothelial cell  
5 procoagulant which causes thrombosis and inflammation.

#### Methods

Inbred mice of strains CBA/J and DBA/2 were obtained from Iffa Credo, France. C57B1/6J and DBA/2 mice were obtained from the Jackson Laboratories, Bar Harbor, ME. C57B1/6 mice with knockout of the interferon response element IRF-1 were generated  
10 as previously described (Duncan, G.S. et al., J. Exp. Med. 184:2043 (1996)) and bred in at the Ontario Cancer Institute, Toronto. CBA/J mice were maintained in the Paris colony under conventional open-top wire cage conditions with food and water *ad lib* and a 12 hour light-dark cycle. Mice in the Toronto colony were maintained in a barrier facility. Female CBA/J, C57B1/6 or C57B1/6 IRF-/- mice were mated by overnight cohabitation with a  
15 DBA/2J male, and the morning of sighting a vaginal plug was defined as day 0.5 of pregnancy.

Pregnant CBA/J mice were depleted of NK cells by injection of 1 ml rabbit IgG anti-asialoGM1 antibody (Immunocorp, Richmond, VA) ip on day 6.5 of gestation; phosphate buffered saline (PBS) was used as a control as it has been previously shown to be  
20 equivalent to non-immune rabbit IgG (Clark, D.A., Crit. Rev. Immunol. 11:215 (1991)). Macrophage depletion was performed by ip injection of 100 mg/kg silicon dioxide twice a week for 4 weeks prior to mating, as described in Baek, H-S. and J-W. Yoon (J. Virol. 64:5708 (1990)). Affinity-purified rabbit IgG neutralizing antibody to mouse procoagulant (fgl2-prothombinase) was prepared as previously described (Ding, J.W. et al., J. Exp. Med.  
25 (1997); Dackiw, A.I.B. et al., Arch. Surg. 131:1273 (1996)); the mice were given a ip injection of 200  $\mu$ l of a 1/50 dilution of a 5.5 mg/ml preparation of anti-prothombinase or control rabbit antibody each day beginning on day 3.5 of gestation. Hormonal support of pregnancy sufficient to replace ovarian function was provided in some experiments by injecting 6.7 ng 17 $\beta$ -estradiol + 1 mg progesterone in 0.1 ml oil im daily beginning on day 4.5  
30 of gestation (Michael, S.D. et al., Biol. Reprod. 12:400 (1975)). One hundred  $\mu$ l of rat monoclonal IgG2b anti-mouse granulocyte antibody RB6-8C5 (Pharmingen) (Stoppacciaro, A. et al., J. Exp. Med. 178:151 (1993)) or isotype control was injected ip on either day 6.5 or on day 8.5 of pregnancy. TNF- $\alpha$  (6 and R&D Systems) 1000 or 2000 units and/or murine recombinant  $\gamma$ -interferon (6 and R&D Systems) 1000 units was injected ip on day 7.5 of  
35 pregnancy. On day 13.5 of pregnancy, the mice were sacrificed and the number of resorbing and healthy embryos was counted. In some experiments, the uteri were snap frozen, 5 micron sections were cut, and the tissues were stained with rat monoclonal F4/80 antibody (Caltag, Tebu, France) to macrophages. Briefly, tissue sections were incubated with a 1/30 dilution



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of F4/80 in PBS for 30 minutes, and binding was detected using peroxidase-streptavidin with biotin-labelled anti rat IgG2b second antibody (Serotec) (Karchhach, M. et al., Biol. Reprod. 45:860 (1991)).

- Four to ten mated mice per treatment group were used. The significance of differences in the pooled resorption rate was tested by  $\chi^2$  of Fisher's Exact test where appropriate.

#### Results and Discussion

##### NK and macrophage depletion and fetal loss

- Experiment 1, Table 4, shows that ip injection of TNF- $\alpha$  boosted the fetal loss rate of DBA/2-mated CBA/J mice in a dose dependent manner. If the mice had received anti-asialoGM1 antibody treatment, the background rate of fetal loss decreased, as expected (Clark, D.A., Crit. Rev. Immunol. 11:215 (1991); Clark, D.A. et al., Ann. New York Acad. Sci. 626:524 (1990); Chaouat, G. et al., J. Reprod. Fert. 89:447 (1990)), and TNF- $\alpha$  no longer had a significant effect. These data supported the model TNF- $\alpha$   $\rightarrow$  NK  $\rightarrow$  activated NK  $\rightarrow$  kill embryo. To ensure adequate levels of endogenous macrophage-derived TNF- $\alpha$ , we repeated the experiment and added  $\gamma$ -interferon. Experiment 2, shows that  $\gamma$ -interferon alone boosted the fetal loss rate in PBS-pretreated mice to the level achieved with TNF- $\alpha$ , and addition of TNF- $\alpha$  had no significant additional effect. In NK cell-depleted mice,  $\gamma$ -interferon failed to boost fetal losses. This suggested the model  $\gamma$ -interferon  $\rightarrow$  macrophages  $\rightarrow$  activated to produce NC  $\rightarrow$  embryo death was not correct. However, when  $\gamma$ -interferon and TNF- $\alpha$  were administered together, more than 80% of the implanted embryos aborted. This suggested an obligatory synergy/co-dependence; in NK cell depleted mice, TNF- $\alpha$  does not work because the NK cell source of  $\gamma$ -interferon has been eliminated, and  $\gamma$ -interferon fails because macrophages dependent on NK cell-derived  $\gamma$ -interferon, have stopped producing TNF- $\alpha$ , as the ip injected cytokine does not stimulate TNF- $\alpha$  production quickly enough such that both cytokines are present simultaneously. A direct NK or macrophage killing mechanism seemed unlikely to explain fetal losses. To further test this idea, the experiment was repeated using macrophage-depleted mice. Experiment 3, shows that macrophage depletion reduced the fetal loss rate. It can be seen that macrophage depletion had no significant effect on the 80% fetal loss rate produced by injecting TNF- $\alpha$  +  $\gamma$ -interferon. Tissue staining for F4/80 $^{+}$  macrophages confirmed the silica treatment had been effective and the cytokine treatment did not cause a macrophage infiltration (data not shown). TNF- $\alpha$  +  $\gamma$ -interferon may act synergistically to suppress production of essential gestational hormones by the ovary (Teranova, P.R. and V.M. Rice, Reprod. Immunol. 37:50 (1997)) and such an inhibition could cause fetal losses (Deansly, R., J. Reprod. Fert. 35:183 (1973); Kaplanski C. et al., J. Immunol. 158:5435 (1997); Michael, S.D. et al. Biol. Reprod. 12:400 (1975)). However, ovarian inhibition should have caused 100% fetal losses (Deansly, R. J. Reprod. Fert. 35:183 (1973)). Further, when we gave



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hormone replacement therapy as described above (Michael, S.D. et al. Biol. Reprod. 12:400 (1975)), there was no effect on either the background rate of fetal loss or the high rate of fetal loss produced by 2000 u TNF- $\alpha$  + 1000 u  $\gamma$ -interferon (35/41, 86% N=5 control group versus 37/45, 82% N=5 cytokine-treated group, not statistically different).

- 5                   *Cytokine-triggered fetal loss in IRF1-/- mice.* TNF- $\alpha$  and  $\gamma$ -interferon act synergistically to induce apoptosis in human trophoblast cell cultures (Yui, J. et al., Placenta 15:819 (1994)). The results shown in Table 4 could be explained by a direct apoptotic action on trophoblast. However, the cytokine CSF-1 is present in vivo, and this may abrogate the apoptotic effects of TNF- $\alpha$  and  $\gamma$ -interferon (Yui, J. et al., Placenta 15:819 (1994); Pollard, J.W. et al., Nature 330:484 (1987)). To test for a direct effect on trophoblast in mice, IRF1 -/- females were mated to DBA/2 (+/+) males. Here the fetal trophoblast expresses IRF1 but maternal tissues do not. As shown in Table 5, pregnant IRF1-/- females had low background fetal loss rates and were completely resistant to TNF- $\alpha$  +  $\gamma$ -interferon. The C57BL/6 (+/+) female conspecific with the IRF1-/- mice also had a low resorption rate, but aborted dramatically when the cytokine treatment was given. These data indicated the cytokines act on the mother and not on trophoblast to induce fetal losses.

*Anti-flg2 prothrombinase antibody blocks fetal losses, and granulocytes contribute to the process of endothelial disruption.*

- Since neither macrophages nor NK cells seemed necessary for TNF- $\alpha$  +  $\gamma$ -interferon to act, the most logical target appeared to be the vascular endothelial cell. These cytokines stimulate surface expression of pro-coagulant (flg2-prothrombinase, which is distinct from tissue factor) and the subsequent clotting process is known to lead to ischemic damage in a variety of inflammatory disease models such as hepatitis and endotoxic shock (Ding, J.W. et al., J. Exp. Med. (1997); Darki A.P.B. et al., Arch. Surg. 131:1273 (1996); Levi, M. et al., J. Clin. Invest. 93:114 (1994)).

- The results of treatment of DBA/2-mated CBA/J mice with antibody to flg2 prothrombinase are shown in Table 6. Treatment with antibody to flg2 prothrombinase reduced the background risk of fetal loss from 38% to 4.5%; the reduction is statistically significant at  $p < 0.001$ . The frequency of chromosome abnormalities in mouse embryos is 4% (Smith, W.B. et al., J. Immunol. 157:360 (1996)).

- Table 6 also shows that treatment with antibody to flg2 prothrombinase markedly reduced the fetal loss risk induced by TNF- $\alpha$  +  $\gamma$ -interferon from 67% to 13% ( $p < 0.001$ ). There is no statistically significant difference between the fetal loss risk in mice which received TNF- $\alpha$  +  $\gamma$ -interferon and antibody to flg2 prothrombinase (13%), and the fetal loss risk in mice which did not receive TNF- $\alpha$  +  $\gamma$ -interferon, but received antibody to flg2 prothrombinase (4.5%). Therefore, antibody to flg2 prothrombinase almost completely prevented fetal loss induced by TNF- $\alpha$  +  $\gamma$ -interferon.



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Thus, it has been found in this study that neither NK cells nor macrophages are required for fetal loss, that cytokine-dependent fetal loss in CBA x DBA/2 mice is mediated by the procoagulant fgl2 prothrombinase, that the cytokines act on the mother and not on embryonic trophoblast, and that the embryo dies from ischemia due to activation of vascular endothelial cell procoagulant fgl2 which causes thrombosis. The present inventor has demonstrated that treatment of DBA/2-mated CBA/J mice with antibody to fgl2 prothrombinase reduces the background risk of fetal loss and almost completely prevents fetal loss induced by TNF- $\alpha$  +  $\gamma$ -interferon.

#### Example 6

#### 10 Expression of fgl2 prothrombinase using a baculovirus expression system

The example demonstrates that the fgl2 protein may be expressed using a baculovirus expression system.

cDNA of murine fgl2 was subcloned into the vector pBlueBacHis2A. This was then used to generate a recombinant baculovirus by homologous recombination with the wild type baculovirus AcMNPV. By infecting insect cells (Sf9 or High 5) with the recombinant virus, a fusion mfgl2 protein with six histidine residues followed by an enterokinase site at the N-terminus was expressed. The lysate from High 5 cells infected with the recombinant virus was analyzed for the presence of mfgl2 protein by Western blot using the polyclonal rabbit anti-mouse fgl2 antibody. Uninfected High 5 cells and those infected with wild type AcMNPV were used as controls in all studies. The conditions for protein expression were optimized (see Results section) and the fusion protein was purified using the Probond resin, which contains nickel for binding the histidine residues.

Recombinant viruses containing the mfgl2 gene sequence were screened by PCR and selected as putative clones. Pure viral clones were obtained after several rounds of plaque purification. Western blot analysis was performed using polyclonal rabbit anti-mfgl2 antibody to demonstrate expression of mfgl2 fusion protein. Preliminary experiments were performed to determine the optimal conditions for protein expression. Amounts of protein expressed by Sf9 and High 5 insect cells were compared. High 5 cells expressed greater quantities of protein compared to Sf9 cells. Recombinant protein production was detectable by 48 h and reached maximal levels at 72 h, remaining at the same level for up to 5 days after infection. The time course of mfgl2 expression is shown in Figure 19 MOI = MOI is of 5 or 10 pfu/cell produced similar levels of protein expression. Based on these observations, we decided to infect High 5 cells with virus at an MOI of 5 pfu/cell, and harvested the cells on day 3 post-infection for optimal protein production.

As we mentioned previously, the mfgl2 fusion protein was attached to a polyHis tag at the N terminus. Purification of the protein was performed using the Probond resin under denatured conditions & no success was met when performed under native conditions. Western and Coomassie blue staining were used to detect the expressed



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prothrombinase (Figures 20 and 21 respectively). The protein was observed to have a molecular weight of ~ 60 kDa. Murine fgl2 fusion protein was not detected in the medium.

#### Example 7

##### Functional analyses of the mfgl2 fusion protein

- 5 Functional analyses on the procoagulant activity of the expressed mfgl2 fusion protein prepared in Example 6 were conducted to confirm that the expressed fusion protein acts as a direct prothrombinase, like the native protein.

##### Experimental approach

##### One-stage clotting assay

- 10 This assay for fgl2 prothrombinase was used to directly measure procoagulant activity. High 5 cells infected with recombinant virus expressing the mfgl2 fusion protein were subjected to cycles of freeze thawing. They were then assayed for their ability to accelerate the spontaneous clotting time of recalcified platelet-poor normal human plasma as previously described (Levy & Edgington, 1980). Results were quantitated by comparison with serial dilutions of standard rabbit brain thromboplastin. Activities from cells infected with the recombinant virus were compared with those uninfected and wild-type virus infected, and with the purified protein alone. Proteins involved in the coagulation pathway require a phospholipid bilayer for functional activity; therefore purified protein was reconstituted into the insect cells and macrophages to determine their
- 15 PCA activity. Additional PCA assays were performed with human plasma deficient in coagulation factors II, V, VII, VIII, X, and XII to determine the nature (factor dependence) of the expressed PCA.

##### Prothrombin Cleavage Assay

- To determine if the expressed fusion protein acts as a direct prothrombinase, the prothrombin cleavage assay was performed as previously described (Schwartz et al., 1982). 125I-prothrombin was incubated with High 5 cells, both infected or uninfected with the recombinant virus. Purified mfgl2 fusion protein was also studied to determine its ability to cleave prothrombin. Human Factor Xa in the presence of Russell's viper venom (RVV) was used as a positive control. Samples were run on 10% SDS-PAGE and analyzed by autoradiography for 125I-prothrombin and their cleavage products.
- 25
- 30

##### One-stage clotting assay

- PCA was measured for uninfected cells, wild type- and recombinant virus-infected cells, and the soluble protein (Table 7). Only cells infected with the recombinant virus express PCA, and no activity were detected from the purified soluble protein. This result suggested that the presence of the six histidine residues and the enterokinase site at the N-terminus of mfgl2 fusion protein did not completely affect its clotting ability. The PCA results using factor deficient plasmas are shown in Table 8. The PCA expressed by recombinant virus infected cells was independent of all factors except II (prothrombin)
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which suggests the expressed mfg12 fusion protein acts similarly as the native protein to be a direct prothrombinase.

A second set of clotting assays was done to determine if the addition of soluble mfg12 protein prevents PCA expression by cells infected with recombinant virus. Our results suggested that the soluble protein was incapable of preventing the clotting induced by the cell lysate. Nonetheless, preliminary results from reconstitution of purified protein into insect cells shows partial PCA activity recovery (Table 9).

#### Prothrombin Cleavage Assay

The ability of mfg12 fusion protein to cleave prothrombin to thrombin was examined by the prothrombin cleavage assay. In Figure 22, a single high molecular weight species of intact 125I-prothrombin was noted after incubation with buffer and calcium alone (first panel). Addition of human factor X in the presence of calcium and factor V produced cleavage products corresponding to known derivatives of prothrombin (second panel). Similar products were seen when incubating 125I-prothrombin with homogenates from recombinant virus infected cells (third panel). However, incubation of 125I-prothrombin with uninfected High 5 cell homogenates or purified protein exhibited no prothrombin cleavage (third and fifth panels respectively). These results were consistent with the observations from our one-stage clotting assay. Low molecular weight products were seen when 125I-prothrombin was incubated with homogenates from wild type virus infected cells (data not shown). This might be explained by the expression of protease in wild type infected insect cells (Viakird et al., 1995).

Having illustrated and described the principles of the invention in a preferred embodiment, it should be appreciated to those skilled in the art that the invention can be modified in arrangement and detail without departure from such principles. We claim all modifications coming within the scope of the following claims.

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

Below full citations are set out for the references referred to in the specification and detailed legends for some of the figures are provided.



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**TABLE 1**

Clan	Example	Consensus
SA	chymotrypsin <i>10 families</i>	H D G.SG
SB	Subtilisin <i>1 family</i>	D HGT GTS.....G
SC	Carboxypeptidase <i>5 families</i>	C G.S D H
SE	B lactamase <i>4 families</i>	S..K S.N D
SF	Iex A <i>3 families</i>	SM G KR
SG	Omptin <i>5 families</i>	not identified



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TABLE 2

Clan	Example	Consensus
CA	Papain <i>3 families</i>	CW H N.W W
CB	Picornains <i>4 families</i>	H C(G)
CC	HC-proteinase <i>2 families</i>	GYCY VDH HV
-	many uncharacterized families	



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**TABLE 3**

PRIMER	SEQUENCE	5' POSITION	bp
			2(A+T)+4(G+C)
HUFLP1	GCA AAC AAT GAA ACA GAG GAA A	100	80
HUFLP2	ATT GGC CTA TTA GAT AAC GAA TAC	1399	84
HUFLP3	AAC GGA GAC CCA GGC AGA AAC	349	86
HUFLP4	CTT CGG GAG CTG AAT AGT CAA	243	62
HUFLP5	GAC AGC AAA GTG GCA AAT CTA	553	60
HUFLP6	TTC TGG TGA AGT TGG TGC TCC	832	84
HUFLP7	CAA AAG AAG CAG TGA GAC CTA CA	893	66
HUFLP8	TTA TCT GGA GTG GTG AAA AAC TT	1125	62
HUFLP9	TGA CCA AGA GTA AGG AAA TGA	908	58
HUFLP10	TGA CTG TAT TTG TTC TTG GGT G	639	62
HUFLP11	TTC TGG GAA CTG TGG GCT GTA	1134	64
HUFLP12	CCA GGT TCA TCT TTA CAG T	43	54
HUFLP13	AAT CAC TCT GTT CAT TCC TCC	1353	60
HUFLP14	GAA ATA ATA TGC ATT GAA A	-179	36
HUFLP14R	AAC GCA CAG GAA GAG GAG A	-86	58
HUFLP15	TTG ACA TCC TTT GAG ATA T	1459??	60
HUFLP16	ATG GGG CAT TGG GGA GC	-427	56
HUFLP17	GGC TAT CTC CTC TTC CTG T	-118	58
HUFLP18	TGA GCT ATG CCA GTG TCT GT	-755	60
HUFLP19	CAA GCG TAG TAT ACC AAA T	-288	52
HUFLP20	AAG GCA GGA AAG AGG AAC	-961	54
HUFLP21	GAC AAA GGA ATA GAA AGT AGC	-601	58
HUFLP22	CAG GGC AAA AAT CTA AAT G	-1092	52
HUFLP23	GCC CAG AGA GCA GGT AGA A	-883	60
HUFLP24	CCA GCC AGG GTT GAA ATA	3' end	54
HUFLP25	GCC CTG TCA GTC ATT TTG	promoter not used	64
HUFLP26	AAA AAC CTA CCA GTA GTC T	3' end	52
HUFLP28	TTG GGG TGA CAT TAT GC	2389	50
HUFLP 29	TGA GCA GCA CTG TAA AGA TG	16	58
HUFLP30	GTG GCT TAA AGT GCT TGG GT	1350	60



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**TABLE 4**

Role of asialoGM1+ NK cells and macrophages in fetal loss

Expt.	Day 6.5 treatment	Day 7.5 treatment	Day 13.5 assay		
			N <sub>a</sub>	resorptions / total	% fetal losses
5	1. PBS	PBS	8	23/56	41%
	PBS	1000 u TNF- $\alpha$	8	43/60	72% <sup>b</sup>
	PBS	2000 u TNF- $\alpha$	8	57/64	89% <sup>c</sup>
	anti-asialoGM1	PBS	8	10/59	19% <sup>d</sup>
	anti-asialoGM1	1000 u TNF- $\alpha$	8	12/63	16% <sup>d</sup>
	anti-asialoGM1	2000 u TNF- $\alpha$	8	12/55	22% <sup>e</sup>
	2. PBS	PBS	16 <sup>f</sup>	43/101	43%
	PBS	1000 u IFN- $\gamma$	16	79/93	85% <sup>g</sup>
	PBS	1000 u IFN- $\gamma$ + TNF- $\alpha$ <sup>h</sup>	16	74/89	83% <sup>h</sup>
	anti-asialoGM1	PBS	16	11/71	15% <sup>d</sup>
	anti-asialoGM1	1000 u IFN- $\gamma$	16	12/98	12 % <sup>e</sup>
	anti-asialoGM1	1000 u IFN- $\gamma$ + TNF- $\alpha$ <sup>h</sup>	16	89/104	86% <sup>h</sup>
	3. ctrl	PBS	8	36/88	41%
	ctrl	IFN- $\gamma$ + TNF- $\alpha$	8	65/80	81% <sup>i</sup>
	SiO <sub>2</sub> <sup>k</sup>	PBS	8	14/55	25% <sup>k</sup>
10	SiO <sub>2</sub>	IFN- $\gamma$ + TNF- $\alpha$	8	52/65	80% <sup>i</sup>

## Footnotes to Table 4:

- a) N represents number of pregnant mice per group.
- b) Significant increase in fetal loss rate,  $P < 0.005$  by c2.
- c) Significant increase in fetal loss rate compared to PBS control,  $P < 0.005$  by c2 ;
- 15 d) Significant difference compared to lower dose of TNF- $\alpha$ ,  $P < 0.05$ .
- d) Significant reduction in fetal loss rate by anti-asialoGM1 antibody compared to PBS control,  $P < 0.005$  by c2.
- e) No significant boosting of fetal loss rate compared to PBS injected anti-asialoGM1-treated group.
- 20 f) Result from 2 independent experiments giving same result have been pooled.
- g)  $\gamma$ -interferon (IFN- $\gamma$ ) significantly boosted fetal loss rate.  $P < 0.005$  by c2
- h)  $\alpha$ -interferon (IFN- $\alpha$ ) significantly boosted fetal loss rate.  $P < 0.005$  by c2



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- h) TNF- $\alpha$  was given at 1000 u and 2000 u in separate experiments with IFN- $\gamma$  and gave similar result; the data have been pooled for ease of presentation. The fetal loss rate was significantly boosted  $P < 0.005$  by  $\chi^2$ .
- i) Untreated CBA/J female mice mated to DBA/2 males.
- 5 j) 1000 u IFN- $\gamma$  + 2000 u TNF- $\alpha$  significantly boosted fetal loss rate,  $P < 0.005$  by  $\chi^2$ .
- k) CBA/J mice injected twice a week for 4 weeks with 100 mg/kg silicon dioxide before mating significantly reduced fetal loss rate,  $P < 0.05$  by  $\chi^2$ .



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**TABLE 5**

Cytokine-triggered resorption in C57Bl/7 IRF+/+ and IRF-/- mice

	Mating combination	Day 7.5 treatment	N	Day 13.5 assay	
				resorptions /total	% fetal losses
	IRF1+/+ X +/+ <sup>a</sup>	nil	5	3/39	7.7%
5	IRF1+/+ X +/+	IFN- $\gamma$ + TNF- $\alpha$	4	16/22	73% <sup>b</sup>
	IRF1-/- X +/+ <sup>c</sup>	nil	7	3/57	5.3%
	IRF1-/- X +/+	IFN- $\gamma$ + TNF- $\alpha$ <sup>d</sup>	9	5/76	6.5%

a) Normal C57Bl/6 females (+/+) mated to DBA/2 males (+/+).

b) Significant increase in fetal loss rate:  $P < 0.001$ , Fisher's Exact test.

c) Female C57Bl/6 mice homozygous for a defective IRF gene (IRF1 -/-) were mated to normal DBA/2 males (+/+).

d) 1000 u IFN- $\gamma$  and 2000 u TNF- $\alpha$  was injected



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**TABLE 6**

Antibody to fg12 prothombinase prevents fetal losses in CBA/J X DBA/2 mice

	Pretreatment group	Day 7.5 treatment	N	Day 13.5 assay	
				resorptions /total	% fetal loss
5	Control Rabbit IgG	nil	8	21/56 <sup>a</sup>	38%
	Control Rabbit IgG	IFN- $\gamma$ + TNF- $\alpha^b$	8	48/55	87% <sup>c</sup>
	Rabbit IgG anti-fg12	nil	9	3/66	4.5% <sup>d</sup>
	Rabbit IgG anti-fg12	IFN- $\gamma$ + TNF- $\alpha^b$	9	9/68	13% <sup>e</sup>

a) Result from two independent experiments which gave the same result.

10 b) 1000  $\alpha$  IFN- $\gamma$  and 2000  $\alpha$  TNF- $\alpha$  was injected ip.

c) Significant increase in fetal loss rate  $P < 0.001$  compared to no cytokine control group, Fisher's Exact test.

d) Significant reduction in spontaneous fetal loss rate  $P < 0.001$  compared to no cytokine control group, Fisher's Exact test.

15 e) Significant reduction in fetal loss rate  $P < 0.001$  compared to cytokine-treated controls, Fisher's Exact test. No significant difference compared to anti-fg12-treated mice which did not receive an injection of cytokines.



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**TABLE 7**

Expression of procoagulant activity in High 5 cells infected with recombinant *AcMNPV*

PCA		
	Time (sec)	Milliunits/2x10 <sup>6</sup> cells
High 5 cells	> 240	<1
5 High 5 cells + Wild type <i>AcMNPV</i>	126±5	9±3
High 5 cells + Recombinant <i>AcMNPV</i>	76±3	319±73
Purified protein (3 µg)	220	<1





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**TABLE 8**

Effect of magulation factor-deficient plasmas on PCA expression

PCA

Plasma	Time (sec)	Milliunits/ $2 \times 10^6$ cells
Normal	72 $\pm$ 4	418 $\pm$ 104
Deficient in:		
Factor VII	54 $\pm$ 4	1624 $\pm$ 406
Factor X	105 $\pm$ 5	40 $\pm$ 14
Factor V	67 $\pm$ 2	592 $\pm$ 89
Factor II	>240	<1



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**TABLE 9**

The procoagulant activity after constitution of the purified protein

PCA		
	Time (sec)	Milliunits (mU/2x10 <sup>6</sup> cells)
H5	>240	<1
Purified protein (3 µg)	220	<1
5 H5 + purified protein (3 µg)	42	3634
H5 + recombinant virus (RV)	89	127
H5 cells + RV + Purified protein (20 ng)	57	1254
10 H5 cells + RV + Purified protein (100 ng)	54	1543
H5 cells + RV + Purified protein (3 µg)	50	2053
15 H5 cells + RV + Purified protein (3µg)	34	6434



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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Levy, Gary
- (ii) TITLE OF INVENTION: Methods of Modulating Immune Coagulation
- (iii) NUMBER OF SEQUENCES: 4
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: BERESKIN & PARR
  - (B) STREET: 40 King Street West
  - (C) CITY: Toronto
  - (D) STATE: Ontario
  - (E) COUNTRY: Canada
  - (F) ZIP: M5H 3Y3
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Gravelle, Micheline
  - (B) REGISTRATION NUMBER: 40,261
  - (C) REFERENCE/DOCKET NUMBER: 9579 006
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: (416) 364-7311
  - (B) TELEFAX: (416) 361-1308

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 4630 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (vi) ORIGINAL SOURCE:
  - (A) ORGANISM: Homo sapiens
- (xii) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 439 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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 20              25              30
Glu. Arg. Ala. Lys. Asp. Val. Cys. Pro. Val. Arg. Leu. Glu. Ser. Arg. Gly. Lys.
 35              40              45
Cys. Glu. Glu. Ala. Gly. Glu. Cys. Pro. Tyr. Gln. Val. Ser. Leu. Pro. Pro. Leu.
 50              55              60
Thr. Ile. Glu. Leu. Pro. Lys. Gln. Phe. Ser. Arg. Ile. Glu. Glu. Val. Phe. Lys.
 65              70              75              80
Glu. Val. Gln. Asn. Leu. Cys. Gln. Ile. Val. Asn. Ser. Leu. Lys. Lys. Ser. Cys.
 85              90              95
Gln. Asp. Cys. Lys. Leu. Gln. Ala. Asp. Asp. Asn. Gly. Asp. Pro. Gly. Arg. Asn.
100              105              110
Gly. Leu. Leu. Leu. Pro. Ser. Thr. Gly. Ala. Pro. Gly. Glu. Val. Gly. Asp. Asn.
115              120              125
Arg. Val. Arg. Gln. Leu. Glu. Ser. Glu. Val. Asn. Lys. Leu. Ser. Ser. Glu. Leu.
130              135              140
Lys. Asn. Ala. Lys. Glu. Glu. Ile. Asn. Val. Leu. His. Gly. Arg. Leu. Glu. Lys.
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Ile. Tyr. Lys. Asp. Cys. Ser. Asp. Tyr. Tyr. Ala. Ile. Gly. Lys. Arg. Ser. Ser.
210              215              220

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 225 230 235 240  
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 260 265 270  
 Ala Gly Phe Gly Asn Leu Arg Arg Glu Phe Trp Leu Gly Asn Asp Lys  
 275 280 285  
 Ile His Leu Leu Thr Lys Ser Lys Glu Met Ile Leu Arg Ile Asp Leu  
 290 295 300  
 Glu Asp Phe Asn Gly Val Glu Leu Tyr Ala Leu Tyr Asp Gln Phe Tyr  
 305 310 315 320  
 Val Ala Asn Glu Phe Leu Lys Tyr Arg Leu His Val Gly Asn Tyr Asn  
 325 330 335  
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 340 345 350  
 Leu Lys Phe Phe Thr Thr Pro Asp Lys Asp Asn Asp Arg Tyr Pro Ser  
 355 360 365  
 Gly Asn Cys Gly Leu Tyr Tyr Ser Ser Gly Trp Trp Phe Asp Ala Cys  
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 Leu Ser Ala Asn Leu Asn Gly Lys Tyr Tyr His Gln Lys Tyr Arg Gly  
 385 390 395 400  
 Val Arg Asn Gly Ile Phe Trp Gly Thr Trp Pro Gly Val Ser Glu Ala  
 405 410 415  
 His Pro Gly Gly Tyr Lys Ser Ser Phe Lys Glu Ala Lys Met Met Ile  
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## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 5403 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: other nucleic acid

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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AACACTATTT	AACTCTCTCT	AACTCTCTCT	AACTCTCTCT	AACTCTCTCT	2580
ACCTTTCTCT	ATGGCTCTCT	ATGGCTCTCT	ATGGCTCTCT	ATGGCTCTCT	2640
ATAGTACCTA	TAACTTTCTA	TAACTTTCTA	TAACTTTCTA	TAACTTTCTA	2700
CTTATCACTA	ATGACCAAGA	ATGACCAAGA	ATGACCAAGA	ATGACCAAGA	2760
AATATGACAT	GACATATAAC	GACATATAAC	GACATATAAC	GACATATAAC	2820
TAAAGCTTTT	CACTCTCTCT	CACTCTCTCT	CACTCTCTCT	CACTCTCTCT	2880
GAATCTTTTA	TGAACTTTT	TGAACTTTT	TGAACTTTT	TGAACTTTT	2940
ATACTTCTCT	TCTAATCTCT	TCTAATCTCT	TCTAATCTCT	TCTAATCTCT	3000
CACCTCTCTT	CTAATCTCT	CTAATCTCT	CTAATCTCT	CTAATCTCT	3060
AAATAATATA	TTCAGGATTA	TTCAGGATTA	TTCAGGATTA	TTCAGGATTA	3120
ACTGGAATCA	TTTAACTCTA	TTTAACTCTA	TTTAACTCTA	TTTAACTCTA	3180
GUACTGAAGG	CTGACATTTA	CTGACATTTA	CTGACATTTA	CTGACATTTA	3240
TGCTGATTTT	ATCTTAGGAG	ATCTTAGGAG	ATCTTAGGAG	ATCTTAGGAG	3300
GTTATGAGAG	TAAAGCTCTT	TAAAGCTCTT	TAAAGCTCTT	TAAAGCTCTT	3360
AACTCTCTCT	TGCTGAGTAC	TGCTGAGTAC	TGCTGAGTAC	TGCTGAGTAC	3420
CAAGCTCTCT	CTGATATCTA	CTGATATCTA	CTGATATCTA	CTGATATCTA	3480
AGTGATTTGG	AGAGTTTCTT	AGAGTTTCTT	AGAGTTTCTT	AGAGTTTCTT	3540
TTACTACTCT	TGCTGCTTAA	TGCTGCTTAA	TGCTGCTTAA	TGCTGCTTAA	3600
TTGATACAC	CACTCTCTCT	CACTCTCTCT	CACTCTCTCT	CACTCTCTCT	3660
TCTATCAGAG	CCAGGTTCTT	CCAGGTTCTT	CCAGGTTCTT	CCAGGTTCTT	3720
TGTTGATCTT	CAAGTCTCTT	CAAGTCTCTT	CAAGTCTCTT	CAAGTCTCTT	3780
CCCTTTCTCT	CTCTCTCTCT	CTCTCTCTCT	CTCTCTCTCT	CTCTCTCTCT	3840
ACCTCTCTCT	AGGAGGAGG	AGGAGGAGG	AGGAGGAGG	AGGAGGAGG	3900
GCTTTGAGGT	CTACTCTGAC	CTACTCTGAC	CTACTCTGAC	CTACTCTGAC	3960
GCCTTTGAGG	CAGCACCAGC	CAGCACCAGC	CAGCACCAGC	CAGCACCAGC	4020
ACCTTGAGAG	ACAATTTTGG	ACAATTTTGG	ACAATTTTGG	ACAATTTTGG	4080
AAATGATTTT	GAGAACTACT	GAGAACTACT	GAGAACTACT	GAGAACTACT	4140
ATCAGTTTTA	TCTGCTTAA	TCTGCTTAA	TCTGCTTAA	TCTGCTTAA	4200
CCAGGCTAGG	GGATGCTTCT	GGATGCTTCT	GGATGCTTCT	GGATGCTTCT	4260
CAACCTCTCT	CAAGGAGGAG	CAAGGAGGAG	CAAGGAGGAG	CAAGGAGGAG	4320



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CAGGCTGCTG GPTTGATTCA TGTCTCTCTG CCATTTTAA TGGCAATAT TACCACCAGA 4380
AATCAGAGG TGTCTGTAAT GGGATTTTCT GGGGCACTG GCTTGCTATA AACCAAGGAC 4440
ACCGAGGCTG CTACAACTCC TCTTTCANAC AGGCCAAGAT GATGATTAGG CCCAAGGATT 4500
TCAAGCCATA AATTGCTAGT GTTCATCTCT CTGGGCACTG ACTATCTAAC ACCAGGATGA 4560
ATTCTTTCAG CCGTTTACCA TATVTCCTAG TTTATATTC TTTCTATGG CTAAACATTT 4620
CCTTTAAGG TTTACAGCTT TTAGAATAAA GCTGAAAGG TCTAAAAAA CTTCTATGTT 4680
GCTTTTATAT GAGGAATGCT TCAAGCCACT CCAATATTG ACATTTATAC ATTATAATTC 4740
CAAAACCTTT CATTTTATTT AGTTGAAAG TTTCCTAATA TTTTATTAT TTTTATAATA 4800
AAACTAAAT TATTGAGCA GUTAGATTCT ATATACGAA CTTTATTTT CACTAGGCTT 4860
AAATATCAC ATTTGAGAA ATACCACTCC TTCCAGGTAC AACTGAAAGC CAAGACTCT 4920
ACTATTTAT TCTCTCTAAC AAGAACTTA AGCATTTTAG TTCTCAGAA GAAGGGCAGC 4980
GATGGGATTC GGGGCCAGGG ACAATATGTA TAGCTAAATG TATTCATCTA ATGCAAAATA 5040
TGGCAATAAA ATACCTAAAA ATCTCTTACG ATAATATAT TCTCTTCCC CTCCAAATGA 5100
AAATAATAT TACCTCTTAG ACTTTGGTTT AGTGTAAT CACTTACTGT TTATAGCCTG 5160
TTAGACCGGG ATACAAAAGC TCTTTATTC TCTCTTTTC CTCTCTCTTC ACAAATCTTT 5220
GTATATTAAG GTGCTAGACT ACTGTAAGGT TTCTTTGGGG AAAGGCTAGG TAAGGGAAAT 5280
CACACTGGTT TATATTTTGA AAGCTAATTC TAAATCAAA GCAATACTCT TCTGAGGAG 5340
TCAACCTTCT ACGAGCTGA CTTTCTAGA CCAATGTAT TTATTAGGAT GAATTTGGGA 5400
ATT 5403

```

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 432 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```

Met Arg Leu Pro Gly Trp Leu Trp Leu Ser Ser Ala Val Leu Ala Ala 1
5 10 15
Cys Arg Ala Val Glu Glu His Asn Leu Thr Glu Gly Leu Glu Asp Ala 20
25 30
Ser Ala Glu Ala Ala Cys Pro Ala Arg Leu Glu Gly Ser Gly Arg Cys 35
40 45
Glu Gly Ser Gln Cys Pro Phe Gln Leu Thr Leu Pro Thr Leu Thr Ile 50
55 60
Gln Leu Pro Arg Gln Leu Gly Ser Met Glu Glu Val Leu Lys Glu Val 65
70 75 80

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Arg Thr Leu Lys Gln Ala Val Asp Ser Leu Lys Lys Ser Cys Glu Asp  
 85 90 95  
 Cys Lys Leu Gln Ala Asp Asp His Arg Asp Pro Gly Gly Asn Gly Gly  
 100 105 110  
 Asn Gly Ala Glu Thr Ala Glu Asp Ser Arg Val Gln Glu Leu Glu Ser  
 115 120 125  
 Gln Val Asn Lys Leu Ser Ser Glu Leu Lys Asn Ala Lys Asp Gln Ile  
 130 135 140  
 Gln Gly Leu Gln Gly Arg Leu Glu Thr Leu His Leu Val Asn Met Asn  
 145 150 155 160  
 Asn Ile Glu Asn Tyr Val Asp Asn Lys Val Ala Asn Leu Thr Val Val  
 165 170 175  
 Val Asn Ser Leu Asp Gly Lys Cys Ser Lys Cys Pro Ser Gln Glu His  
 180 185 190  
 Met Gln Ser Gln Pro Val Gln His Leu Ile Tyr Lys Asp Cys Ser Asp  
 195 200 205  
 His Tyr Val Leu Gly Arg Arg Ser Ser Gly Ala Tyr Arg Val Thr Pro  
 210 215 220  
 Asp His Arg Asn Ser Ser Phe Glu Val Tyr Cys Asp Met Glu Thr Met  
 225 230 235 240  
 Gly Gly Gly Trp Thr Val Leu Gln Ala Arg Leu Asp Gly Ser Thr Asn  
 245 250 255  
 Phe Thr Arg Glu Trp Lys Asp Tyr Lys Ala Gly Phe Gly Asn Leu Glu  
 260 265 270  
 Arg Glu Phe Trp Leu Gly Asn Asp Lys Tle His Leu Leu Thr Lys Ser  
 275 280 285  
 Lys Glu Met Ile Leu Arg Ile Asp Leu Glu Asp Phe Asn Gly Leu Thr  
 290 295 300  
 Leu Tyr Ala Leu Tyr Asp Gln Phe Tyr Val Ala Asn Glu Phe Leu Lys  
 305 310 315 320  
 Tyr Arg Leu His Ile Gly Asn Tyr Asn Gly Thr Ala Gly Asp Ala Leu  
 325 330 335  
 Arg Phe Ser Arg His Tyr Asn His Asp Leu Arg Phe Phe Thr Thr Pro  
 340 345 350  
 Asp Arg Asp Asn Asp Arg Tyr Pro Ser Gly Asn Cys Gly Leu Tyr Tyr  
 355 360 365  
 Ser Ser Gly Trp Trp Phe Asp Ser Cys Leu Ser Ala Asn Leu Asn Gly  
 370 375 380  
 Lys Tyr Tyr His Gln Lys Tyr Lys Gly Val Arg Asn Gly Ile Phe Trp  
 385 390 395 400  
 Gly Thr Trp Pro Gly Ile Asn Gln Ala Gln Pro Gly Gly Tyr Lys Ser  
 405 410 415  
 Ser Phe Lys Gln Ala Lys Met Met Ile Arg Pro Lys Asn Phe Lys Pro  
 420 425 430

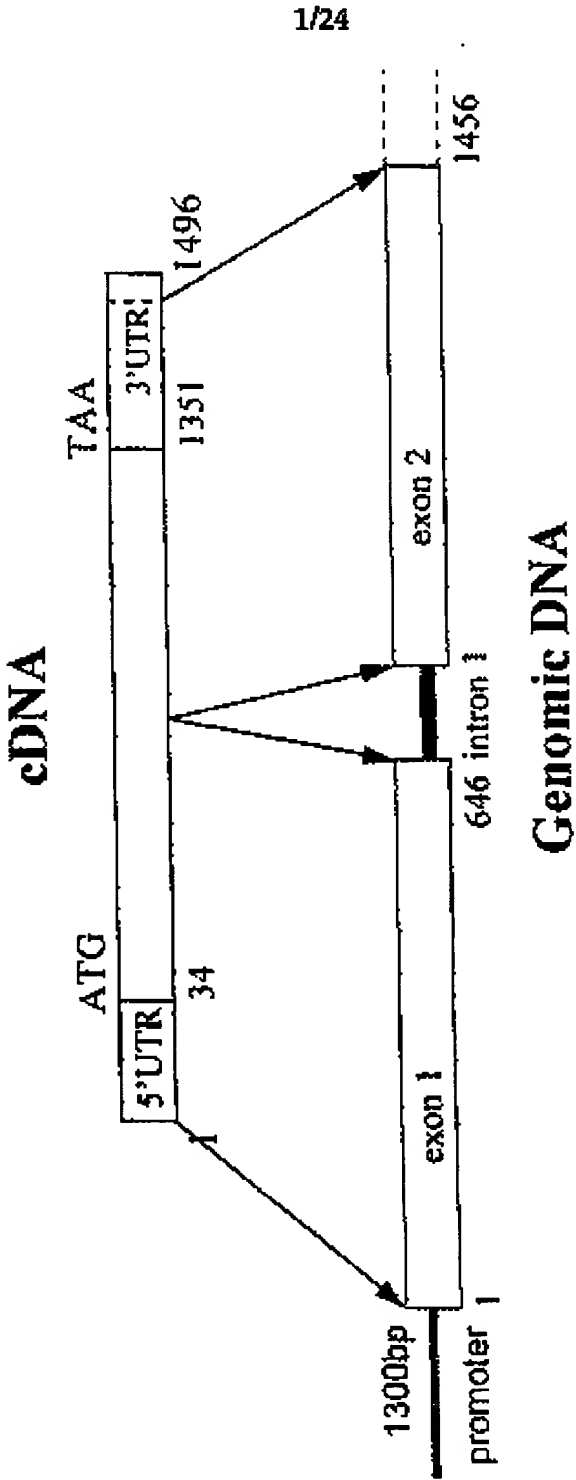


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FIGURE 1





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2/24**FIGURE 2**

	10	20	30	40	50	
MOUSE-X1.DNA	1 ATGAGGCTTC	CTGGTTGSTT	CTGAGTGAAT	TCGCGCTCC	TCGCTGCCTG	50
HUMAN-X1.DNA	1 ATGAGGCTTC	CTAGCTGCTA	CTGAGTGAAT	TCAGCTGCTC	TTGCCACCTA	50
	60	70	80	90	100	
MOUSE-X1.DNA	51 CCGAGC---G	GTCGAGGAGE	ACAACCTGAC	TCAGGCGCTG	GAGGATGCCA	100
HUMAN-X1.DNA	51 CCGT7TTTTC	CTTGTGCTCA	ACAATGAATC	ACAGGAAATT	AAAGATGAAA	100
	110	120	130	140	150	
MOUSE-X1.DNA	101 GCGCCGAGGC	TGCCTGCCCC	GCGAGGCTGG	AGGCGAGCGG	GAGGTGCGAG	150
HUMAN-X1.DNA	101 GAGCAAGGA	TGTCTGCCCC	GTGAGACTAG	AAAGCAGAGG	GAAATGCGAA	150
	160	170	180	190	200	
MOUSE-X1.DNA	151 GGG---GCC	AGTGGCCCTT	CCAGTCCACC	CTGCCCCACG	TGACCATCCA	200
HUMAN-X1.DNA	151 GAGGCGAGGG	AGTGGCCCTA	CCAGGTAAAG	CTGCCCCCTT	TGACTATTCA	200
	210	220	230	240	250	
MOUSE-X1.DNA	201 GCTCCCGCGG	CAGCTTGCCA	GCA7GGAGGA	GCTGCTCAA	GAACTCCGA	250
HUMAN-X1.DNA	201 GCTCCCGAAG	CAATTCAGCA	GGATCGAGGA	GCTGCTCAA	GAACTCCGA	250
	260	270	280	290	300	
MOUSE-X1.DNA	251 CCTCAAGGA	AGCAGTGGAC	AGTCTGAAGA	AATCCTGCCA	GGACTGTAA	300
HUMAN-X1.DNA	251 AACTCAAGGA	AATCGTAAAT	AGTCTGAAGA	AATCCTGCCA	AGACTGCAAG	300
	310	320	330	340	350	
MOUSE-X1.DNA	301 TTGCAAGGTG	ACGACCATCG	AGATCCCGGC	GGGATGAGAG	GG-----	350
HUMAN-X1.DNA	301 CTGCAAGGTG	ATGACCAAGG	AGACCCAGGC	AGAAACGGAC	TGTGTTTACC	350
	360	370	380	390	400	
MOUSE-X1.DNA	351 -AAT---GGA	GC---AGACA	CAGCCGAGGA	CAGTAGAGTC	CAGGAACCTG	400
HUMAN-X1.DNA	351 CAGTACAGGA	GCTCCGGGAG	AGGTTGGTGA	TAACAGAGTT	AGACAATTAG	400
	410	420	430	440	450	
MOUSE-X1.DNA	401 AGAGTCAGGT	GAAACAAGCT	TCCTCAGAGC	TGAAGAATGC	AAAGGACCTAG	450
HUMAN-X1.DNA	401 AGAGTGAGGT	TAACAAGCTG	TCCTCTGAGC	TAAAGAAATC	CAAGGAGGAG	450
	460	470	480	490	500	
MOUSE-X1.DNA	451 ATCCAGGCGC	TCCAGGCGCG	CCTGGAGACG	CTCCATCTCG	TAAATATGAA	500
HUMAN-X1.DNA	451 ATCAATCTAC	TTCATGGTGG	CCTGGAGAAC	CTCAATCTTG	TAAATATGAA	500
	510	520	530	540	550	
MOUSE-X1.DNA	501 CAACATTGAG	AACTACGCGG	ACAACAAAGT	GGCAATCTTA	ACCGTGTGTG	550
HUMAN-X1.DNA	501 CAACATAGAA	AAATATGTTG	ACACCAAGT	GGCAATCTTA	ACATTTCTTT	550
	560	570	580	590	600	
MOUSE-X1.DNA	551 TCAACAGTTT	GGATGGCAAC	TGTTCCAAGT	GTOCCAGCCA	AGAACACATG	600
HUMAN-X1.DNA	551 TCATATAGTT	GGATGGCAAA	TGTTCCAAGT	GTOCCAGCCA	AGAACAAATA	600
	610	620	630	640	650	
MOUSE-X1.DNA	601 CAGTCACAGC	CGG.....	.....	.....	.....	650
HUMAN-X1.DNA	601 CAGTCACGTC	CAG.....	.....	.....	.....	650



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3/24**FIGURE 3**

	10	20	30	40	50	
MOUSE-X2.DNA	1	TTCACATCT	AATATACAA	GATTGTTCG	ACCACTACCT	GCTAGGAAGG 50
HUMAN-X2.DNA	1	TTCACATCT	AATATATAA	GATTGTCTG	ACTACTACGC	AATAGGCAAA 50
	60	70	80	90	100	
MOUSE-X2.DNA	51	AGAAGCAGT	GGGCTACAG	AGTTACCCCT	GATCAGAGAA	ACAGCAGCTT 100
HUMAN-X2.DNA	51	AGAAGCAGT	AGACCTACG	AGTTACCCCT	GATCCCAAAA	ATAGTAGCTT 100
	110	120	130	140	150	
MOUSE-X2.DNA	101	TGAGSTCTAC	TGTGACATG	AGACCATGG	TGBAGGCTGG	ACGGTGTCTG 150
HUMAN-X2.DNA	101	TGAAGTTTAC	TGTGACATG	AGACCATGG	GGGAGGCTGG	ACAGTGTCTG 150
	160	170	180	190	200	
MOUSE-X2.DNA	151	AGGCTCCCT	TGATGGCAG	ACCAACTTCA	CCAGAGAGTG	GAAGAGCTAC 200
HUMAN-X2.DNA	151	AGGCTCCCT	CGATGGCAG	ACCAACTTCA	CCAGAACATG	GCAAGACTAC 200
	210	220	230	240	250	
MOUSE-X2.DNA	201	AAAGCCGCT	TTGCAAACT	TGACGAGAA	TTTTGGTTGG	CCAACGATAA 250
HUMAN-X2.DNA	201	AAAGCAGCT	TTGCAAACT	CAGAGGGAA	TTTTGGTTGG	GGAACGATAA 250
	260	270	280	290	300	
MOUSE-X2.DNA	251	AATTCATCT	CTGACCAAG	GTAAGGAAAT	GATTTTGAAG	ATAGATCTTG 300
HUMAN-X2.DNA	251	AATTCATCT	CTGACCAAG	GTAAGGAAAT	GATTCCTGAG	ATAGATCTTG 300
	310	320	330	340	350	
MOUSE-X2.DNA	301	AAGACTTTAA	TGGTCTCACA	CTTTATGCC	TGTATGATCA	GTTTATGTG 350
HUMAN-X2.DNA	301	AAGACTTTAA	TGGTGTGGAA	CTATATGCC	TGTATGATCA	GTTTATGTG 350
	360	370	380	390	400	
MOUSE-X2.DNA	351	GCTAATGAA	TTCTCAAAAT	CCCATACAC	ATCGGTAACT	ACAAATGGCAG 400
HUMAN-X2.DNA	351	GCTAATGAG	TTCTCAAAAT	TCCCTTACAC	GTTGGTAACT	ATAATGGCAG 400
	410	420	430	440	450	
MOUSE-X2.DNA	401	GGCAGGGGAT	GCCTTGCTT	TGAGTCGACA	CTACAACTAT	GACCTGAGGT 450
HUMAN-X2.DNA	401	AGCTCGAGAT	GCATTACCT	TGACAAACA	TTACAACTAC	GATCTGAAGT 450
	460	470	480	490	500	
MOUSE-X2.DNA	451	TTTTCACAC	CCAGACAGA	GACAACTAT	GCTACCCCTC	TGGGAATCTT 500
HUMAN-X2.DNA	451	TTTTCACAC	TCCAGATAA	GACAACTAT	GATATCTCTC	TGGGAATCTT 500
	510	520	530	540	550	
MOUSE-X2.DNA	501	GGGCTCTATT	ACAGCTCAG	CTGGTGGTT	GATTCATGTC	TCTCTGCCAA 550
HUMAN-X2.DNA	501	GGGCTGTACT	ACAGTTCAG	CTGGTGGTT	GATGCATGTC	TTTCTGCCAA 550
	560	570	580	590	600	
MOUSE-X2.DNA	551	CTTAAATGG	AAATATTACC	ACAGGAAATA	CAAGGTGTC	CGTAATGGGA 600
HUMAN-X2.DNA	551	CTTAAATGG	AAATATTATC	ACCAAAATA	CAAGGTGTC	CGTAATGGGA 600
	610	620	630	640	650	
MOUSE-X2.DNA	601	TTTCTGTGG	CACCTGGCT	GGZATAAAC	AGSCACAGCC	AGTGGGTAC 650
HUMAN-X2.DNA	601	TTTCTGTGG	TACCTGGCT	GGGTAAAGT	AGSCACAGCC	TGTTGGGTAC 650
	660	670	680	690	700	
MOUSE-X2.DNA	651	AAGTCTCTCT	TCAACAGGC	CAAGATGATG	ATTAGGCCCH	AGGATTTCAA 700
HUMAN-X2.DNA	651	AAGTCTCTCT	TCAAGAGGC	TAAGATGATG	ATCAGACCCA	AGCACTTTAA 700
	710	720	730	740	750	
MOUSE-X2.DNA	701	GCCATAA...	.....	.....	.....	750
HUMAN-X2.DNA	701	GCCATAA...	.....	.....	.....	750



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10	20	30	40	50	60
ATCACTCTGT	TCATTCCTCC	AGGTATTGCT	TATCTAATAG	GGCAATTAA	TCCTTCAGCA
70	80	90	100	110	120
CTTTAGATA	TGCTTGTTT	CATATTTTTC	ATACTAATA	AATGCTTGT	TTTATATTT
130	140	150	160	170	180
TCATAGCTTA	AAATGATGT	CTGACGCTA	GGTCTTTATG	CTACACAGCA	TTTGAATAA
190	200	210	220	230	240
AGCTGAATA	CAATGCATTT	TAAAGGATTC	CTTGTTTGT	ATCTGTATT	CCATGAACA
250	260	270	280	290	300
CTTCACAGCA	ATTAGCAATA	TTGAGAAATA	TACATTAGAT	TTACAATTC	TTTAATTTCT
310	320	330	340	350	360
ATTGAACCT	TTTCTATTGC	TTGTATTACT	TGCTGTATTT	AAAAAATA	TGTTGCTCG
370	380	390	400	410	420
GTGTGCTAGC	TCACGCTGT	AATTCACACA	CTTGGGATG	TCAGGCTAG	CAGATCACTT
430	440	450	460	470	480
GGGTCAAGCA	GTTCGAGACC	AGCTTGCCA	AACATGTGAA	ACGCTGCTTC	TATTAAAA
490	500	510	520	530	540
ACAAAAATA	GCCGCGCATG	GTGGACATG	CCTGTAATCC	TAGTACTTC	GGAGGCTGAG
550	560	570	580	590	600
GCAAGAGAT	CGCTTGAAAC	TGAAGAGAG	AGGTGCGAT	GAGCCAGAA	TGAGCCACTG
610	620	630	640	650	660
CACCTCAGCA	TGGGTGACAG	AGAAACTCT	GTCTCAACA	AAAAAATA	AAATTTATT
670	680	690	700	710	720
CAGTAGGNTG	GATTCACAC	AAATATATCT	GTATTTGGCC	CATGATTTAA	GCACATCTGA
730	740	750	760	770	780
AGGTATATCA	CTCTTTTCAG	GCTATATAA	TTTGGGTAT	CTTCATCTG	AGACAAACTG
790	800	810	820	830	840
AATCTATATC	ATTTACTTTC	CACAGAGCA	ACCTACAGC	ATTTTGGTTC	CCAGACTAAG
850	860	870	880	890	900
GGAACTAATA	TCTATATAAT	TAACTTGTT	CATTATCAT	TCATGAATA	TAAATCTT
910	920	930	940	950	960
GTCAATTAAT	CGTTTAAAA	ATGTGCTAGC	ATATGTCTC	CCAAAAAGC	ATTCAGAGAG
970	980	990	1000	1010	1020
CATGTAACT	GTGAGAGCA	GGTTTAAAG	GTATTTCTT	TATAGTTTAT	AACTCCCTAG
1030	1040	1050	1060	1070	1080
ATGTTTGATG	TTGAATCTG	CTTTAATCT	AA		

3'UTR of hlg12. The A at position 1 corresponds to position 1354 on the cDNA.

## FIGURE 4



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5/24FIGURE 5

	10	20	30	40	50	
MOUSEPRO.AMI	1	AVLPGSTYLS	AVLPGSTYLS	AVLPGSTYLS	AVLPGSTYLS	50
HUMANPRO.AMI	1	AVLPGSTYLS	AVLPGSTYLS	AVLPGSTYLS	AVLPGSTYLS	50
	60	70	80	90	100	
MOUSEPRO.AMI	51	-GAGCCTPGLT	LTPTTTCCTA	LGSMSTYLS	STRTTTCCTA	100
HUMANPRO.AMI	51	ZAGPCTYV6	LTPTTTCCTA	STRTTTCCTA	STRTTTCCTA	100
	110	120	130	140	150	
MOUSEPRO.AMI	101	AAATTCCTA	GAGCCTPGLT	STRTTTCCTA	STRTTTCCTA	150
HUMANPRO.AMI	101	AAATTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	150
	160	170	180	190	200	
MOUSEPRO.AMI	151	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	200
HUMANPRO.AMI	151	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	200
	210	220	230	240	250	
MOUSEPRO.AMI	201	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	250
HUMANPRO.AMI	201	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	250
	260	270	280	290	300	
MOUSEPRO.AMI	251	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	300
HUMANPRO.AMI	251	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	300
	310	320	330	340	350	
MOUSEPRO.AMI	301	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	350
HUMANPRO.AMI	301	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	350
	360	370	380	390	400	
MOUSEPRO.AMI	351	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	400
HUMANPRO.AMI	351	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	400
	410	420	430	440	450	
MOUSEPRO.AMI	401	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	450
HUMANPRO.AMI	401	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	STRTTTCCTA	450





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6/24**FIGURE 6**

	10	20	30	40	50	
MOUSEPRO.AMI	1	XXLPGWIMLS	SAVLAAAR-A	VSEHNLTEGL	EDASAOACP	ANLEGSGRCE 50
HUMANPRO.AMI	1	XXLAHWYELS	SAVCAIYGF	VVANMETERI	KDERAKDQCP	VXLESGRCE 50
	60	70	80	90	100	
MOUSEPRO.AMI	51	-GSCCPQOLT	LPTLTQLPA	OLGNGSVLK	SVATLKEAVD	SLKKSQDCK 100
HUMANPRO.AMI	51	EAGECPYQVS	LPPLTIQLPK	QFSRTZAVFK	EVQNLKEIVN	SLKKSQDCK 100
	110	120	130	140	150	
MOUSEPRO.AMI	101	LQADDHROP	GHG-----GH	GASTAEDARY	QELESQVTKL	SSELKMAKDQ 150
HUMANPRO.AMI	101	LQADDNGDP	RNGLLPSTG	HPGEVGDNRY	RELESEVTKL	SSELKMAKES 150
	160	170	180	190	200	
MOUSEPRO.AMI	151	QCSLCGRJET	ZHLVMMNIE	NYVDKRVANL	TFVVSIDGK	CSKCPSEQHM 200
HUMANPRO.AMI	151	TWVTHGRJEX	LHLVMMNIE	NYVDSKVANL	TFVVSIDGK	CSKCPSEQEI 200
	210	220	230	240	250	
MOUSEPRO.AMI	201	QSQPVQHLY	KDCSDHYVLG	RRESGAYRYT	PDRNSSEFY	YCMETNGG 250
HUMANPRO.AMI	201	QSQPVQHLY	KDCSDHYAIG	KRSSEYRYT	PDRNSSEFY	YCMETNGG 250
	260	270	280	290	300	
MOUSEPRO.AMI	251	WTVLQANLDS	STNETREMKD	YKAGFGNLK	DFMLGNDKIH	LLFKSKEMJI 300
HUMANPRO.AMI	251	WTVLQANLDS	STNETRETKD	YKAGFGNLK	DFMLGNDKIH	LLFKSKEMLI 300
	310	320	330	340	350	
MOUSEPRO.AMI	301	RIDLEDFNGL	ELYALYDQFY	VAVEFLKYRL	HVGNYNCTAG	DALRTSRYNY 350
HUMANPRO.AMI	301	RJOLEDFNGL	ELYALYDQFY	VAVEFLKYRL	HVGNYNCTAG	DALRTSRYNY 350
	360	370	380	390	400	
MOUSEPRO.AMI	351	HDLAETTTFD	RHDRIYPSGN	CGLYYSGGW	FDSCTSANLN	GKYYHQKYK 400
HUMANPRO.AMI	351	HDLAETTTFD	RHDRIYPSGN	CGLYYSGGW	FDSCTSANLN	GKYYHQKYK 400
	410	420	430	440	450	
MOUSEPRO.AMI	401	VHNGIFNGTW	PGINQAQPGG	YKSSPKQAKM	MTRPKNFKP*	..... 450
HUMANPRO.AMI	401	VHNGIFNGTW	PGVSEANPGG	YKSSPKQAKM	MTRPKNFKP*	..... 450



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### FIGURE 7

[illegible]



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**FIGURE 8**

	10	20	30	40	50	
MOUSEPRO. DNA	1 TCGGTTTCTGA	TATCATGGA	TC-GAATGAG	AAGGGA-AAG	TAGGAGCCCG	50
HUMANPRO. DNA	1 TAGGCTTCTGA	AGCCAGGCTCT	CCTGAGTATG	CGAGATTAAA	TACAGTCATG	50
	60	70	80	90	100	
MOUSEPRO. DNA	51 AGAGTGCCT	AAGACAA--G	GCATAGGCG	TGTCTGACRA	ATCTCTCATA	100
HUMANPRO. DNA	51 GAGTGTAAA	GAGTCTGCCA	ACATTTTGAG	AATGTGATA	GCATTTGGC-	100
	110	120	130	140	150	
MOUSEPRO. DNA	101 CACACATTTC	CCCTTTGAC	ATTCAGTCTG	TATAGGTTAT	TTCTATAGGA	150
HUMANPRO. DNA	101 TA-AAATTAA	GCGGATATAC	AGAAAGTCA	TAGGAATCA	GGTTAAAGAC	150
	160	170	180	190	200	
MOUSEPRO. DNA	151 GAAAAAAAT	ATTCMAATTC	CTTTGCACT	G-GTACACG	CATGAAGGCT	200
HUMANPRO. DNA	151 ATAAATATGA	GATAGGCTAC	AGAGTCTTTT	AAGTANTACA	ATAAACACTT	200
	210	220	230	240	250	
MOUSEPRO. DNA	201 CAGCAAACTC	AATACGTGT	ATGTCCACTT	CGAGACAGTG	CCAGGCGCAA	250
HUMANPRO. DNA	201 TAG--ATTTT	TGCCCATGTC	A-GTCATTTT	GAAATTAATT	TTAAAGCAAA	250
	260	270	280	290	300	
MOUSEPRO. DNA	251 CATTCAGAC	TTCTCAGATA	GAAAGTGCCT	CTGCTGCTG	-TCTCTGAG	300
HUMANPRO. DNA	251 AABACC---C	TTTTTAACA	AGAACTCTTA	TGAGTGTCA	ATATGCAAA	300
	310	320	330	340	350	
MOUSEPRO. DNA	301 --AATSTGA	CAGAGTAGTT	C---AGTTA	GAATTAAGAG	GCATGAGAGA	350
HUMANPRO. DNA	301 CAAATTAAA	CGAGGTGTTT	TCTTAACCTG	AAGCTGTCTC	TCTTTCTGCT	350
	360	370	380	390	400	
MOUSEPRO. DNA	351 AA--AGCTT	CGGAATCTG	GTIAGAGA--	TATAAATATG	AGAACTGAC	400
HUMANPRO. DNA	351 CTTCAGCCTC	TCAAGGAGAA	GTIAGAAAAC	TATATATCAT	AAAGCTACAT	400
	410	420	430	440	450	
MOUSEPRO. DNA	401 ATGSGGTAC	ACACCTGTGA	TCCTGTGTTT	TAGGATGAG	AGGCAAGAG	450
HUMANPRO. DNA	401 GTTTTGA-AC	AAGCTGATAT	ACCAAGTGGC	CCAGAGAGC-	AGGTAGAGAG	450
	460	470	480	490	500	
MOUSEPRO. DNA	451 ATCAGGAGTT	CAAGGCGAGC	CTGAGCTACT	TGACACCTAG	TCTAATATAA	500
HUMANPRO. DNA	451 ACCAGCG---	TGGAGACAGA	--AAGCA--	-GAGCCCTC-G	CCTGCCAGCC	500
	510	520	530	540	550	
MOUSEPRO. DNA	501 TAGAGATAG	ATTACAGAGT	CCCTTTAACT	AGTACAGACA	AAGAAATTGG	550
HUMANPRO. DNA	501 CTACCTGCTC	AA-AGAAAGG	GCAAGATGCT	TGTAGGCAAG	AGAAATTGAG	550
	560	570	580	590	600	
MOUSEPRO. DNA	551 GTTATCTCTG	GTGAGTTAGC	CTGAATTAAT	TTTTAAGTAA	TAAATCCCT	600
HUMANPRO. DNA	551 GACAGACACT	GGCA--TA-G	CTCAAA-GAT	TCACATTTGA	CCAG-----C	600
	610	620	630	640	650	
MOUSEPRO. DNA	601 TTAATAAGA	AACCTTATGA	G-GTCAGTA	CCACATGAA	CTTAAGAGAG	650
HUMANPRO. DNA	601 TGTGGAAGAT	GACAGTACAA	TTACCAAAAT	GT-CGAAGGG	C--AAAGGAG	650
	660	670	680	690	700	
MOUSEPRO. DNA	651 ACCCCAGCT	CTGAGCTGA	GTGATGGGA	ACCAAGGCA	CTGCCCTAGA	700
HUMANPRO. DNA	651 GC---AGCT	ACGCTTTT--	-TGATG---A	AGGACATTA	TGTCTTTT--	700
	710	720	730	740	750	
MOUSEPRO. DNA	701 TGTGTGAGTG	ACGTGCTTTC	AAGTGTTTTA	ACCACTGAGC	ATTACATAGC	750
HUMANPRO. DNA	701 TAAATGSGTC	TTAGACATTT	AGACATTTAT	AT-AC--ACT	ATGCTACGGA	750
	760	770	780	790	800	
MOUSEPRO. DNA	751 CTGACAGTC	AGGAGAAAC	AGCCGTATTC	TCTGCCAGTY	CTCTTCCCTT	800
HUMANPRO. DNA	751 CAAAGCAAT-	AGAAAGTAGC	A-CTTTTTC	TCTACCTAGTT	TCTTCTCTT	800
	810	820	830	840	850	
MOUSEPRO. DNA	801 TTACAAACAG	ATGAGAGACA	CACACAGAGA	ATCCATTTAA	AGAGGAGACC	850
HUMANPRO. DNA	801 TTTCAGTAG	ATGAGGCAA	ACT-CACCTG	CAATAGTCAG	AAAGCTGTAC	850
	860	870	880	890	900	



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**FIGURE 8 cont'd**

MOUSEPRO. DNA	851	TTTCCTCTGA	TTAGGGCCAA	TTTAACTAC	TTAAGAGTC	ACACAAAGTC	900
HUMANPRO. DNA	851	TTTGTACAC	TTAGAACTT	CTAAAGTTC	TTAAGATTTC	ACCTGAAGT	900
		910	920	930	940	950	
MOUSEPRO. DNA	901	TAGCCTTCAA	AAGGAAACA	GGTCCCAA	----CTA--	-GGGAGGAAA	950
HUMANPRO. DNA	901	CCAACAT-GA	AGAAATACA	GGCTCCCAA	TGCCCAATTC	TAAGAAGAAA	950
		960	970	980	990	1000	
MOUSEPRO. DNA	951	CAGAACTCTT	TCCATTTTCG	TGACATTA-	GTCGGAGAA	GCTCAGAGC	1000
HUMANPRO. DNA	951	AAGGACCTTT	TTCATTTTTC	TACGTTTCT	GATCTATAGA	CAGTTTGGAT	1000
		1010	1020	1030	1040	1050	
MOUSEPRO. DNA	1001	ATTIAGACGT	TCCAACCTTT	TCCCACTAG	TG-----C	ACCAAGT-AT	1050
HUMANPRO. DNA	1001	AACTAGCTCT	TACTTTTAT	CTTAAARAC	TGTSTTCCA	GTGAAGTTAC	1050
		1060	1070	1080	1090	1100	
MOUSEPRO. DNA	1051	ATAATATGNT	ATCTTTTGGG	CACCTGTATT	ACAA-CTCTT	TTTAAACAA	1100
HUMANPRO. DNA	1051	GTATAATAT	TTACTTCAG	CG-TAGTATA	CCAAATTACT	TTAGAAATGC	1100
		1110	1120	1130	1140	1150	
MOUSEPRO. DNA	1101	AAGACTTTC	TTGTGCTTTA	CTAAARAC-C	CA-GACCTG	AATCTTCAT	1150
HUMANPRO. DNA	1101	AAGACTTTTC	TTATACTTCA	TAAATACAT	TATGAAAGTG	AATCTTC--T	1150
		1160	1170	1180	1190	1200	
MOUSEPRO. DNA	1151	ACAAAGCGTG	GCACCCACGG	CACGCTTCT	ATTGTGCTA	GTTTTCAGTG	1200
HUMANPRO. DNA	1151	TGGCTGIGTA	CATTIGACTA	TAAATATTTC	AATGCAVAT	ATTTCATTTG	1200
		1210	1220	1230	1240	1250	
MOUSEPRO. DNA	1201	ACAGGAGATG	ACAGCATTTG	GCTGCTGCG	CTTCTGAGG	ACCTTCTCT	1250
HUMANPRO. DNA	1201	AGAGTAAGTT	ACAGTTTTTG	GCAAACTGCG	TTTGTAGAG	GCTATCTCT	1250
		1260	1270	1280	1290	1300	
MOUSEPRO. DNA	1251	CTTG-TGFG-	GCCTCTGAGA	CT-GTGAATC	AAATGCGCCC	CTCCCTTTCT	1300
HUMANPRO. DNA	1251	CTTCTTGTGC	GTCTCTAATA	CTTGTGATTC	AAAGGCTCCC	ACCTTTTCT	1300
		1310	1320	1330	1340	1350	
MOUSEPRO. DNA	1301	GGGAACACAG	AACGCTGAG	TCAGGCGGCG	CTGCTATTA	AAGCC----	1350
HUMANPRO. DNA	1301	GGGAACACAG	AAGGCTGAG	TCAGGCGGCG	CTGCTATTA	AAGCAGCTCC	1350
		1360	1370	1380	1390	1400	
MOUSEPRO. DNA	1351	---CTCTCTC	AG-----CT	GGCT GCGG	CAGTGCAGG	ATG.....	1400
HUMANPRO. DNA	1351	AGCCCTGCG	ACTCTCTCT	GGGTGAGCAG	CAGTGTAAAG	ATG.....	1400



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**FIGURE 9**

10 20 30 40 50  
 TAGGGTTGGAAGCCAGGTCTCCTGAGTATGCGAGAATAAATACAGTCATG  
 60 70 80 90 100  
 GAAGTGTAAGAGTCTGCCAACATTTTGAGAAATGTGAATAGGATTTGGCT  
 110 120 130 140 150  
 AAAATTAAGGGGATATACAGAAAAAGTCATAGGAAATCAGGTTAAAGACAT  
 TCF1 PEA3  
 160 170 180 190 200  
 AAATATGAGATAGGCTACAGAGTGTTTTAAGTAATACAATAAACATTTA  
 GATA1 NF IL6  
 210 220 230 240 250  
 GATTTTGGCCATGTCAGTCATTTTGAAATTATTTTAAAGCAAAAAAAC  
 NF IL6  
 260 270 280 290 300  
 CCTTTTAAACAAGAAATCTTATGAGATGTCATATGCAAAACAAATTAA  
 310 320 330 340 350  
 AAGGAGGTGGTTTCTCTAACTGAAGCTGTTCTCTTTCTGCGCTTCAGCC  
 TCF1  
 360 370 380 390 400  
 TCTGAAGAGAAAAGTTAGAAAACATTATCATTAAATGCTACATGTTTTGAA  
 NF\_E1  
 410 420 430 440 450  
 CAAGCTGATATACCAAGTGGCCAGAGAGCAGGTAGAAGAAOCAGCGTG  
 BHLH  
 460 470 480 490 500  
 AGACAGAAAGCAAGAGGCCCGCTGCCAGGCTACCTGCAGAAAGAAAGG  
 NF IL6  
 510 520 530 540 550  
 GCAAAGATGCTGTAGGCAAGAGAAGTTCAGGACAGACACTGGCATAGGTC  
 TCF1  
 560 570 580 590 600  
 AAAGATTCACTTTGAGCAGCTGTGGAAGATGACAGTACAATTACCAAAA  
 TCF1 BHLH BHLH  
 E2A  
 610 620 630 640 650  
 TGTCGAAGGGCAAAGGAGGCAGCTACTGGTTTGGATGAAAGACAATTATG  
 TCF1 NF IL6  
 660 670 680 690 700  
 TCCTTTTAAATGGGTCTTAGACATTTAGACATTTATATACACTATGCTAC  
 710 720 730 740 750  
 GGACAAAGGAATAGAAAAGTAGCACTTTTTCTCCACTAGTTTTCTTCTCT  
 TCF1  
 760 770 780 790 800  
 TTTTCAAGTAGATGAAGCAAAGTCAACTGCAATAGTCAGAAAGCTGTAC  
 TCF1 BHLH



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**FIGURE 9 CONT'D**

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      810      820      830      840      850
TTTGTTCACCTTAGAACTTCTAAAAAGTCCTTAAGATTTACCTGAAACG
      TCF1      BHLH
      860      870      880      890      900
CCAACATGAAGAAAATACAGGCTCCCCAATGCCCCATTCTAAGAAGAAAA
      910      920      930      940      950
AGGAOCATTTTCATTTTAGTAACGTTTCTGTTCTATAGACAGTTTGGATA
      960      970      980      990      1000
ACTAGCTCTTACTTTTATCTTTAAAAACTGTTTTTCCAGTGAAGTTAOG
      1010      1020      1030      1040      1050
TATAATTATTTACTTCAAGCGTAGTATACCAAATTACTTTAGAAATGCAA
      HF IL6
      1060      1070      1080      1090      1100
GACTTTTCTTATACTTCATAAAATACATTATGAAGTGAATCTTGTGGC
      NF IL6
      1110      1120      1130      1140      1150
TGTGTACATTTGACTATAATAATTTCAATGCATATTATTTCTATTGAGAG
      BHLH
      1160      1170      1180      1190      1200
TAAGTTACAGTTTTTGGCAAACTGCGTTTGTATGAGGGCTATCTCCTCTTC
      1210      1220      1230      1240      1250
CTGTGCGTTTCTAAAACTTGTGATGCAACGCTCCCACCCTTTCTCTGGGA
      AABS
      1260      1270      1280      1290      1300
ACACAGAAACCGTGACTCAGGCACGTGCCGCTATTAAAGCAGCTCCAGCC
      +1      AP 1      BHLH      TATA box
      1310      1320      1330
CTGCGCACTCCCTGCTGGGTGAGCAGCACTGTAAAGATG

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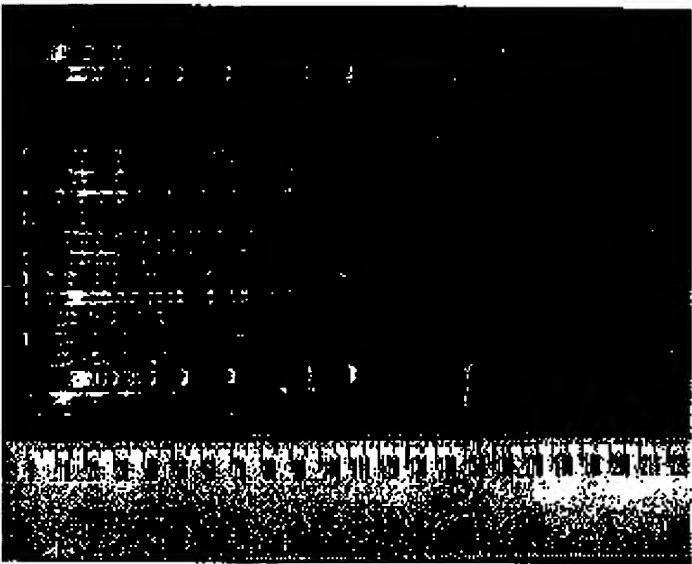


FIGURE 10B

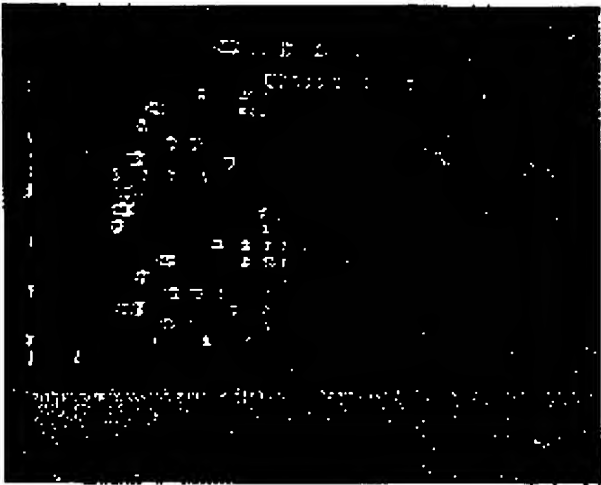


FIGURE 10A



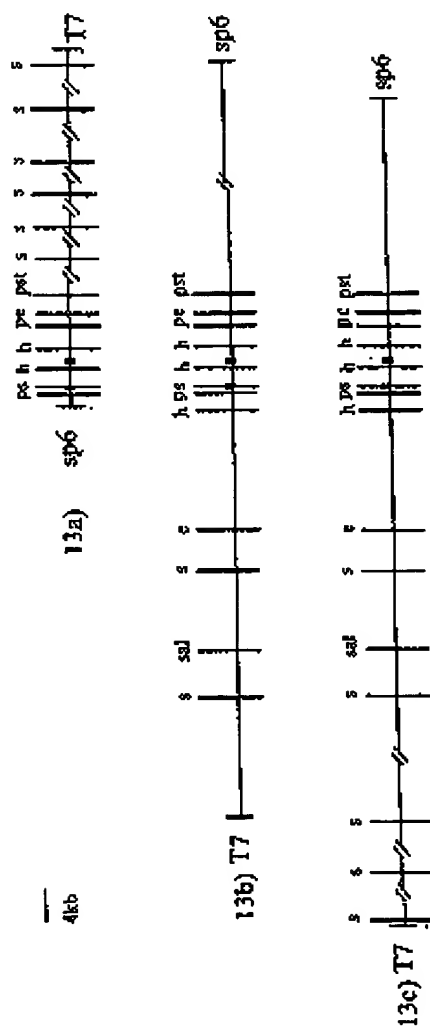
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**FIGURE 11**







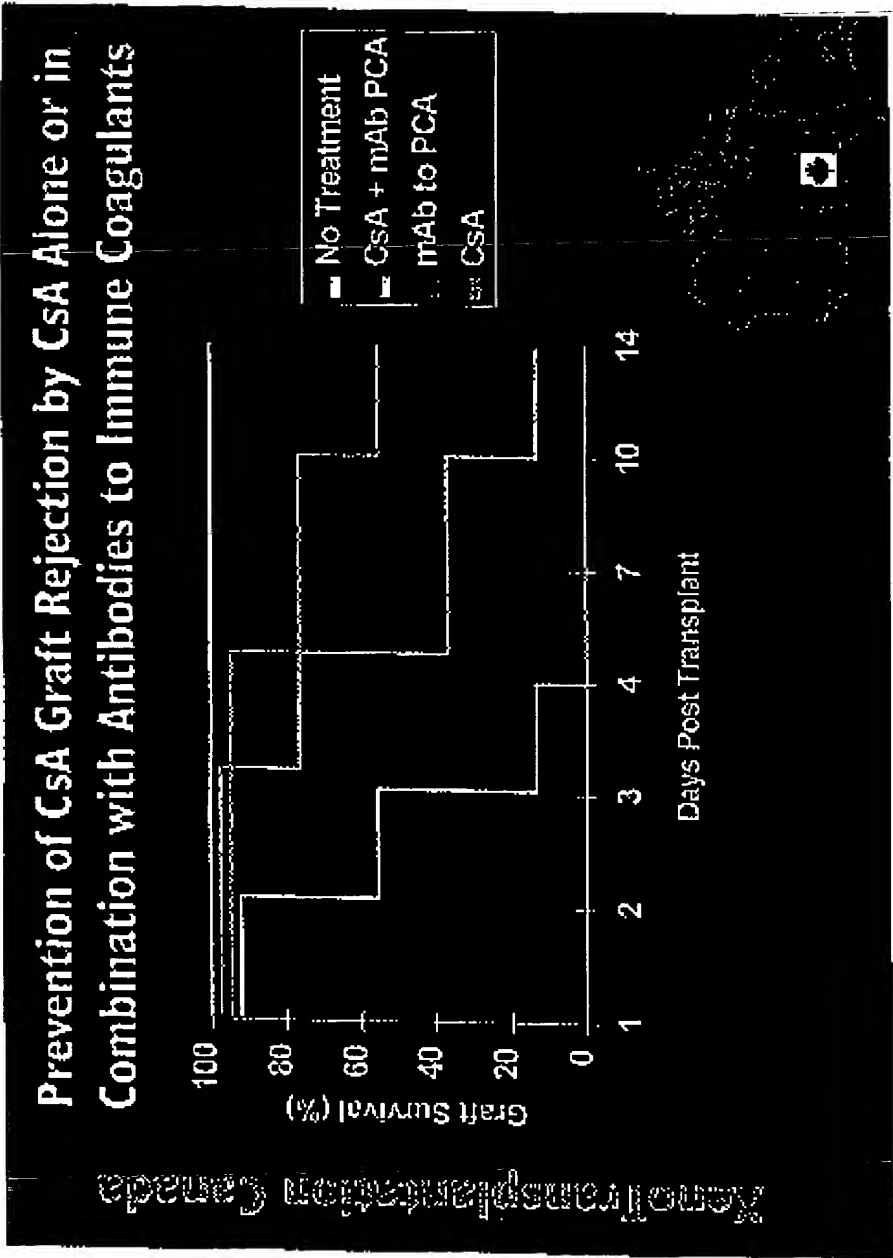
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**FIGURE 12**





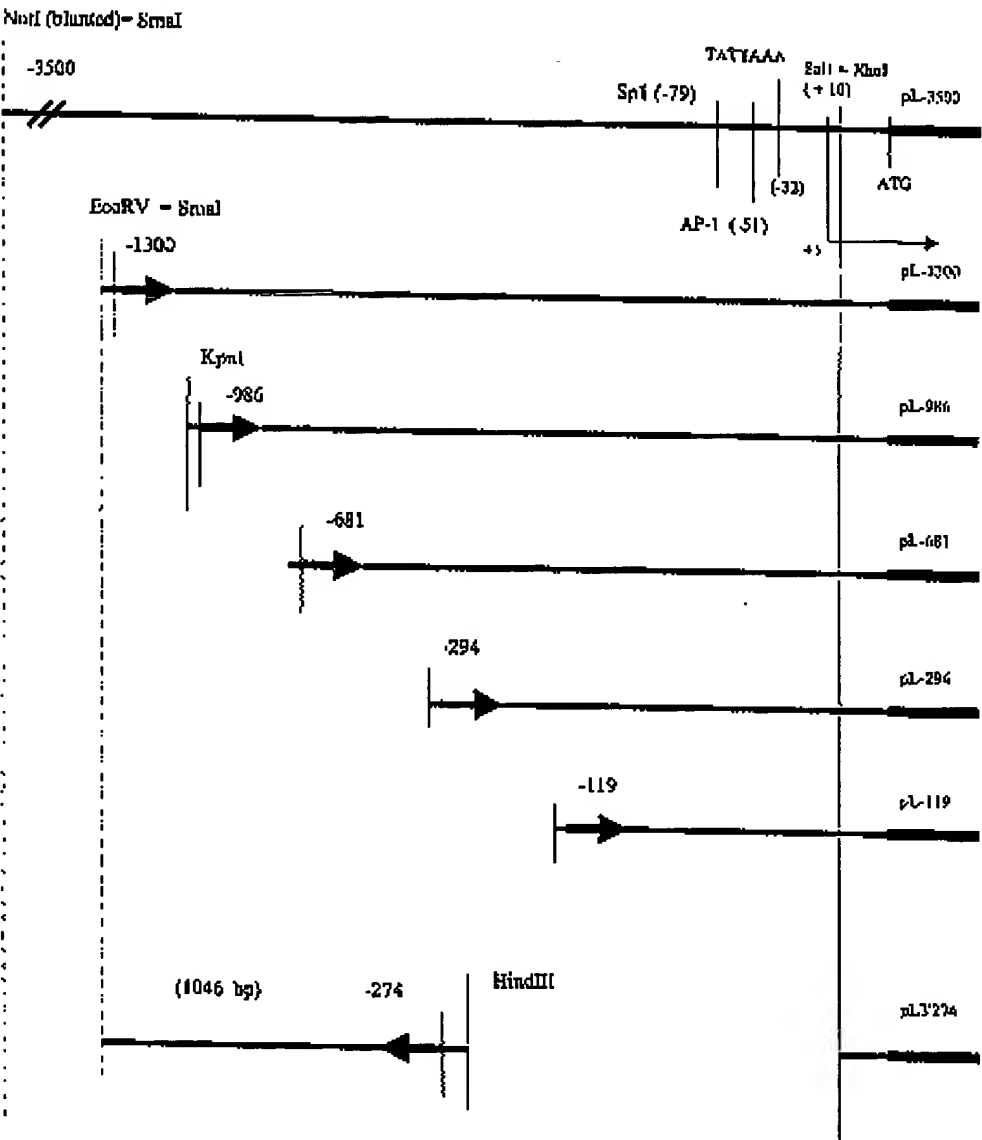
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**FIGURE 13**





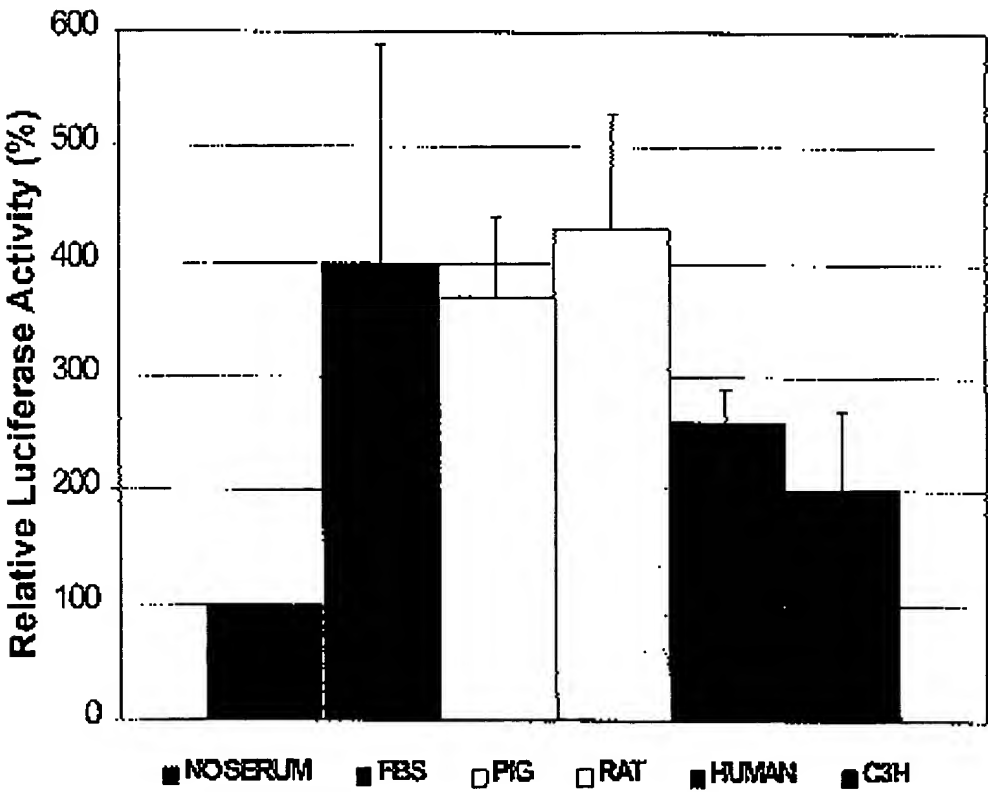
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**FIGURE 14**



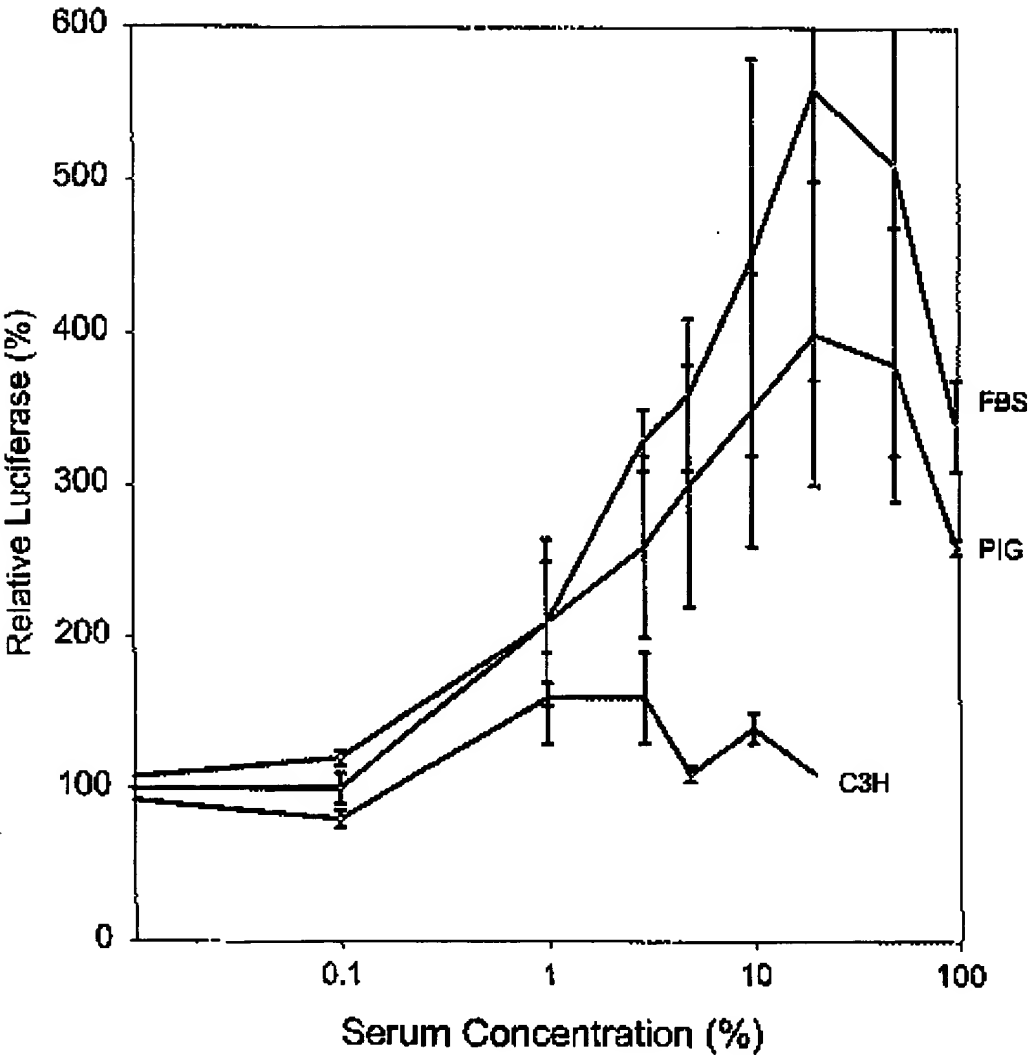


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**FIGURE 15**



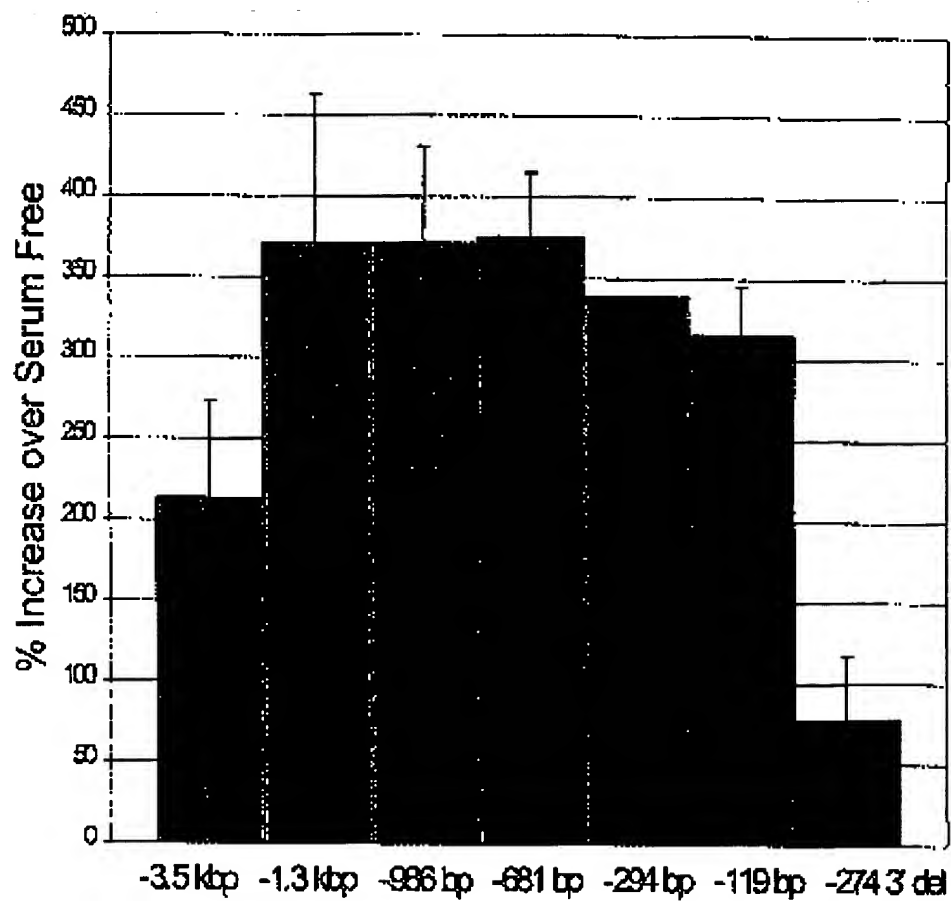


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**FIGURE 16**



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**FIGURE 17**

5'- CCAAGTATAT AATATGGTAT CTTTGGGCA CTGGTATTAC AACTGTTTTT -270  
 TAAACAAAAG ACTTTCCTTG TGCTTTACTA AAAACCCAGA CCGTGAATCT -220  
 TGAATACAAT CCGTGGCAAC CACGOCAGGCATTCTATTGT GCAIAGTTTT -170  
 GACTOACAGG AGATGACAGC ATTTGGCTGC GTGGGCTTGC TGAGGAOCT -120  
 CTCTCCTGT GTGGGCTCTG AACTGTGATGCAAATGGGC CCGCCTTTT -70  
 CTGGGAATC AGAANGCCTG AGTCAGGCGG CCGTGGCTAT TAAAGCGCCT -20  
 GATCAGGCTO GGCTGCCOCA CTCCAAGG--3'

↓  
 +1

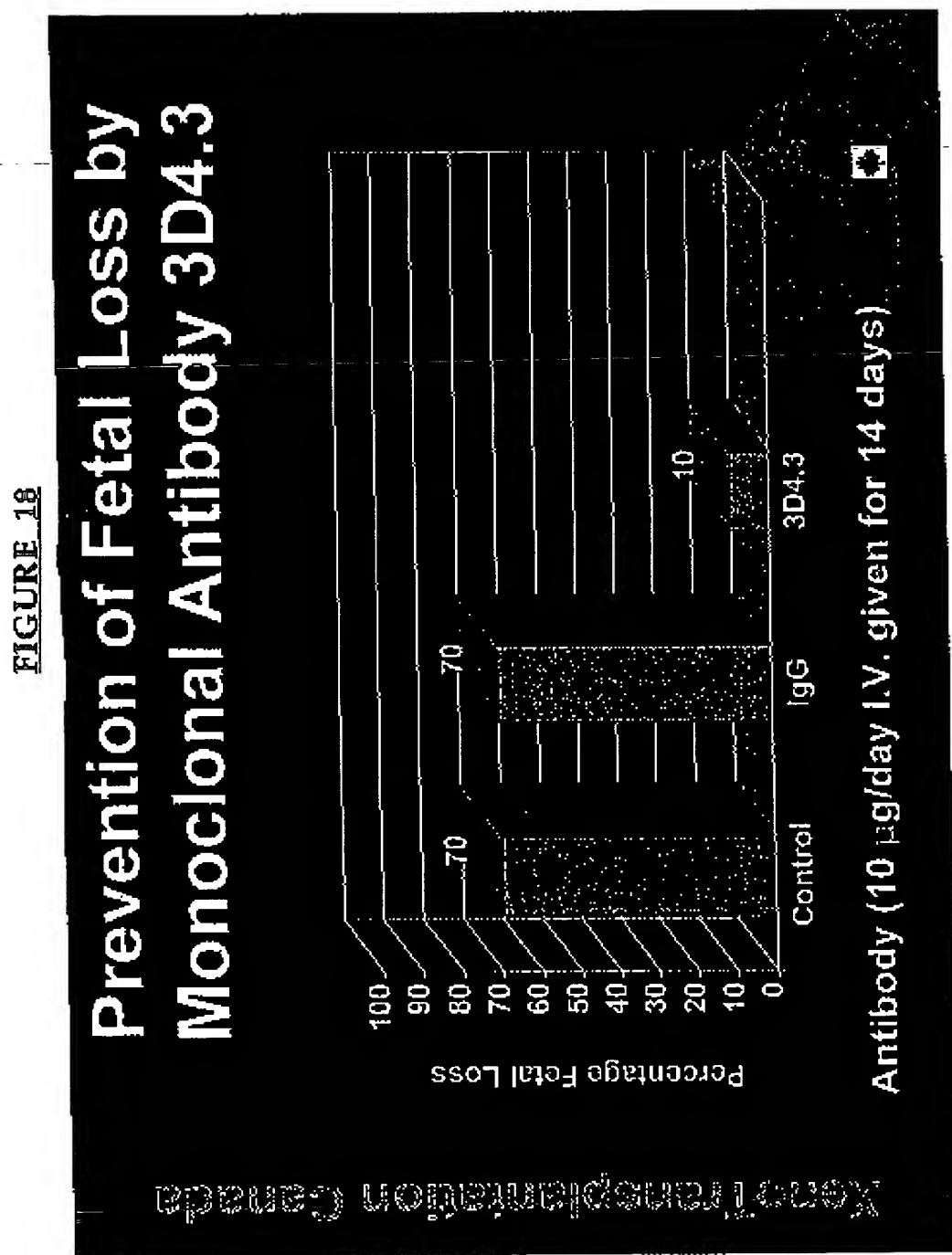


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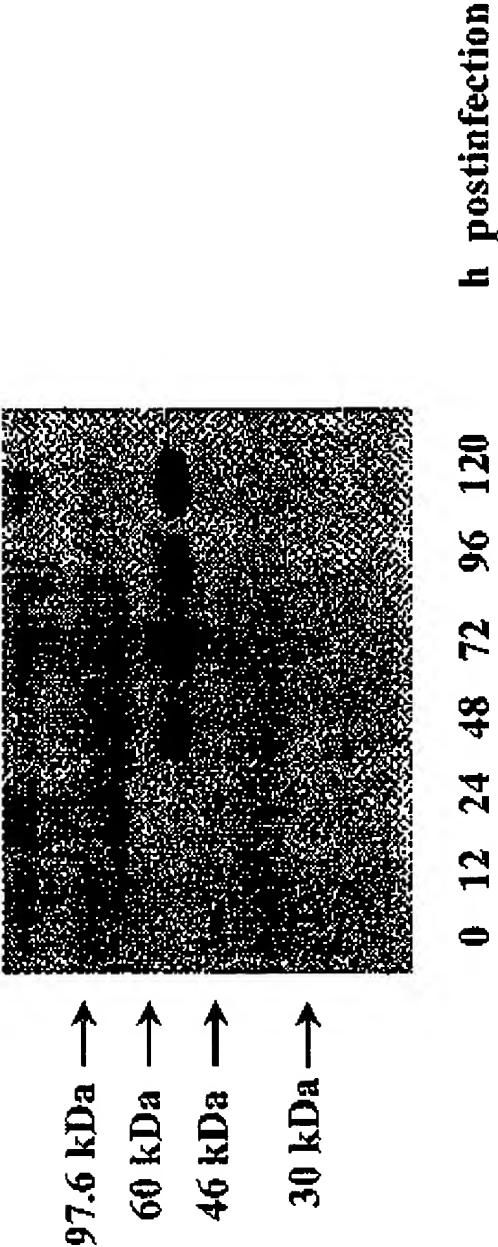
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**FIGURE 19**







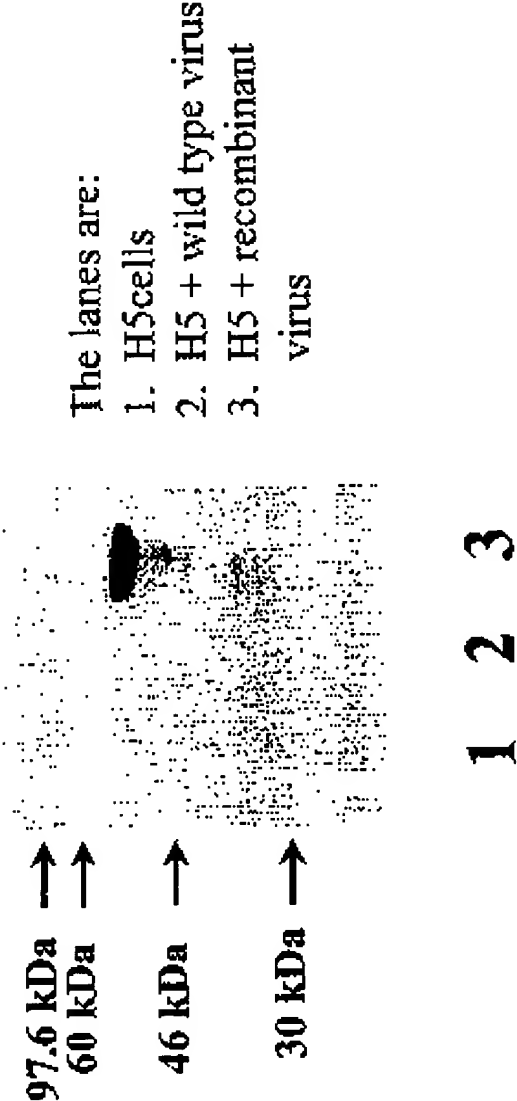
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**FIGURE 20**





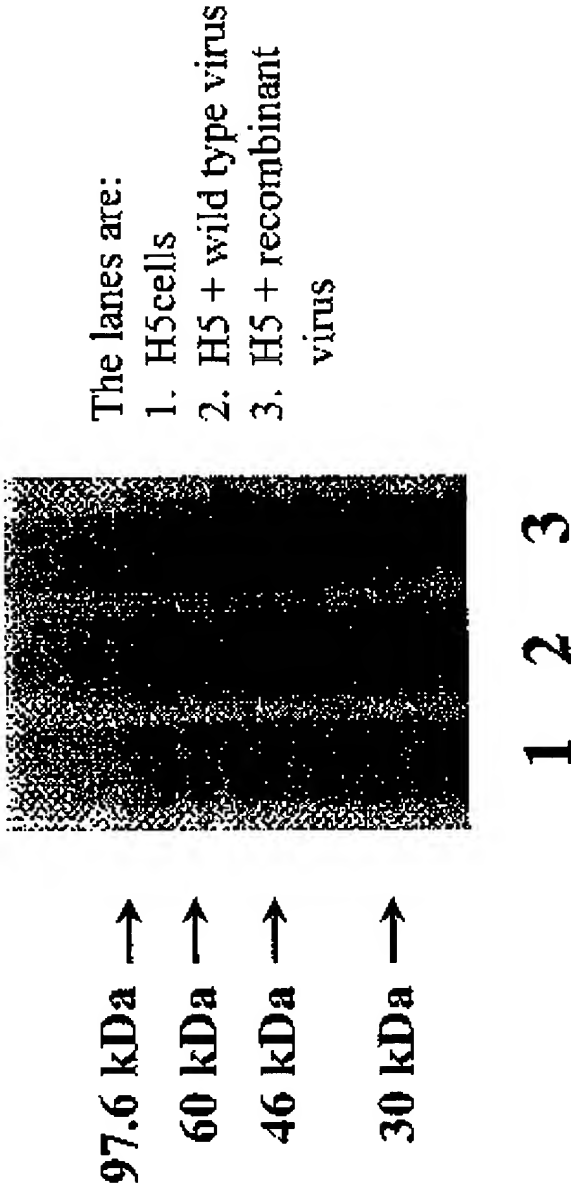
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**FIGURE 21**





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**FIGURE 22**

